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**Investigating Transcriptional Regulation of Viral and Cellular  
Genes by EBV EBNA 2**

By

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Submitted in total fulfilment of the requirements of the degree of

**Doctor of Philosophy**

Department of Biochemistry

School of Life Sciences

University of Sussex

February 2012

## **Declaration**

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award for any other degree.

.....

**UNIVERSITY OF SUSSEX**

**RICHARD PALERMO DPHIL BIOCHEMISTRY**

**INVESTIGATING TRANSCRIPTIONAL REGULATION OF VIRAL AND CELLULAR**

**GENES BY EBV EBNA 2**

**SUMMARY**

Epstein-Barr virus (EBV) is linked to the development of several human malignancies. Epstein-Barr Nuclear Antigen 2 (EBNA 2) is required for the immortalisation and continued proliferation of EBV-infected B-cells. EBNA 2 is a transcriptional regulator of both viral and cellular genes. The viral C promoter (Cp), regulated by EBNA 2, drives transcription of an ~120 kb pre-mRNA that is differentially spliced to generate messages encoding all the other EBNAs required for immortalisation. To study the regulation of Cp-transcript elongation, we used a pair of EBV-infected cell-lines to compare the transcriptional complexes associated with the Cp transcriptional unit and two shorter EBNA 2-regulated viral genes, LMP1 and LMP2A. Interestingly, we found an accumulation of RNA Polymerase II (Pol II) in association with the pausing factors DSIF and NELF at Cp, which were absent at the LMP gene locus. Further experiments revealed that C promoter sequences have a much higher propensity to occlude nucleosome formation, promoting TBP recruitment and Pol II accumulation. We also found high-level recruitment of the Pol II C-terminal domain (CTD) kinase, pTEFb at Cp, increased Pol II Serine 2 CTD phosphorylation and retention at promoter-distal regions. Furthermore pTEFb recruitment at Cp was facilitated by association with the bromodomain protein Brd4 and Pol II pausing. By sustaining a nucleosome-free region and recruiting high levels of pTEFb, Cp elongation appears highly adapted to ensure production of the long EBNA-encoding transcript required to establish and maintain B-cell immortalisation. In additional studies we examined the association of methylated forms of EBNA 2 with viral and cellular genes. EBNA 2 is modified by asymmetric (aDMA) or symmetric (sDMA) arginine di-methylation in the arginine-glycine repeat region. We found that aDMA-modified EBNA 2 preferentially bound promoters to regulate gene expression, implicating this modification as a key regulator of EBNA 2 activity.

## Acknowledgments

I would like to take this opportunity to thank my supervisor Dr Michelle West for her continual patience and endless support throughout my PhD. I would also like to thank the rest of the West-Sinclair lab members, in particular Lina Chen for making my days feel a little bit shorter than they really were.

Thank you to Helen Webb for always being there when I needed you, for helping me obtain my ChIP hat and for inducting me into the very exclusive 1.22 club.

A BIG thank you to my wonderful fiancée Siân for putting up with my work related mood swings and awful sleeping patterns, thanks for supporting me and being the one person there to make me smile when things weren't going in the right direction.

Also many thanks go to my good friend "Chaz" for his ever present I.T support and last but not least I would like to thank my parents for everything they have done and continue to do for me.

## Publications arising from this work

- ❖ **Palermo RD**, Webb HM, West MJ (2011) *RNA Polymerase II Stalling Promotes Nucleosome Occlusion and pTEFb Recruitment to Drive Immortalization by Epstein-Barr Virus*. PLoS Pathogens 7, e1002334
- ❖ Gross H, Barth S, **Palermo RD**, Mamiani A, Hennard C, Zimmer-Strobl U, West MJ, Kremmer E, Grässer FA (2010) *Asymmetric Arginine dimethylation of Epstein-Barr virus nuclear antigen 2 promotes DNA targeting*. Virology 20;397(2):299-310
- ❖ **Palermo RD**, Webb HM, Gunnell A, West MJ (2008) *Regulation of transcription by the Epstein-Barr virus nuclear antigen EBNA 2*. Biochem Soc Trans. 2008 Aug;36 (Pt 4):625-8

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## Abbreviations

AcH3	di-acetylated histone H3
AcH4	di-acetylated histone H4
aDMA-EBNA 2	Asymmetrically di-methylated EBNA 2
BL	Burkitt's Lymphoma
BCR	B-cell receptor
Brd4	Bromodomain-containing protein 4
CAK	CDK-activating kinase
CDK	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation
Cp	C promoter
CR	Conserved region
CTD	C-terminal domain
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DS	Dyad symmetry
DSIF	DRB-sensitivity inducing factor
EBER	EBV-encoded RNA
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
FACT	Facilitates chromatin transcription
FR	Family of repeats
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Germinal centre
GTF	General transcription factor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HEXIM-1	hexamethylene bis- acetamide inducible 1
HIV	Human immunodeficiency virus
HL	Hodgkins Lymphoma
IM	Infectious mononucleosis
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
NELF	Negative elongation factor
PCR	Polymerase chain reaction
PIC	Pre-initiation complex
PID	pTEFb-interacting domain
Pol II	RNA Polymerase II
Poly-RG	Poly-arginine and glycine region
PRC	Polycomb repressive complex
PRMT	Protein arginine N-methyl-transferase
pTEFb	Positive transcription elongation factor b
PTLD	Post-transplant lymphoproliferative disease
Qp	Q promoter
sDMA-EBNA 2	Symmetrically di-methylated EBNA 2
SMN	Survivor motor neuron protein
TAD	Transactivation domain
TAF	TBP-associated factor
TBP	TATA-box binding protein
TCL	Total cell lysate
TR	Travelling ratio
UNPC	Undifferentiated nasopharyngeal carcinoma
Wp	W promoter

# 1. Introduction

## 1.1. Regulation of transcription

Transcription of all protein encoding genes is carried out by RNA Polymerase II (Pol II). The regulation of transcription is important for every cellular process from growth and differentiation to cell survival and apoptosis. Therefore stringent regulation is required to maintain a fully functioning cell. The transcription cycle is tightly regulated and dependent on; the recruitment of Pol II by the general transcription factors; DNA structure and modifications; Pol II CTD phosphorylation and elongation through chromatin.

### 1.1.1. *Attracting Pol II to the promoter*

In eukaryotes Pol II cannot bind to promoters to initiate transcription in the absence of other factors and requires additional multi-protein complexes known as the general transcription factors (GTFs) (Matsui et al., 1980). At gene promoters containing an upstream recognition sequence called the TATA box, initial binding of the GTF TFIID (transcription factor for Pol II D) through the DNA-binding protein TBP (TATA-box Binding Protein) is the first step in pre-initiation complex assembly. In addition, TFIID contains TAFs (TBP-Associated Factors) required for transcriptional regulation. For example TAF<sub>II</sub>250 contains histone acetyltransferase activity (see section 1.1.2.1) (Mizzen et al., 1996). These interactions cause molecular contortion and the bending of DNA, which provides a platform for the recruitment of additional GTFs (Horikoshi et al., 1992, Nikolov et al., 1992). TBP is also recruited through protein-protein interaction to pre-initiation complex assembly at the majority of eukaryotic promoters that do not contain a TATA box (TATA-less promoters) (Latchman, 2008).

The ability of TFIID to bind subsequent GTFs is negatively regulated through associations with the phospho-protein DR1 (Inostroza et al., 1992). TFIIA competes with DR1 for 3 critical lysine residues located within the basic repeat region of TFIID (Buratowski and Zhou, 1992, Lee et al., 1992, Kim et al., 1995). Once DR1 and other TFIID inhibitors are removed, TFIIB binds to TFIID (Kim et al., 1995). TFIIB acts as molecular bridge, permitting the recruitment of the other

GTFs TFIIE, TFIIF, TFIIH and Pol II to form the pre-initiation complex (PIC). TFIIF regulates the association of Pol II and promoter DNA (Hisatake et al., 1993) whilst TFIIE and TFIIH are required for DNA melting and the formation of the open promoter complex through TFIIH-dependent helicase activity (Parvin et al., 1994, Goodrich and Tjian, 1994, Svejstrup et al., 1996). TFIIE actually stimulates both TFIIH kinase and helicase activity to aid in Pol II promoter clearance (Ohkuma and Roeder, 1994, Trigon et al., 1998, Lu et al., 1992, Douziech et al., 2000). TFIIH kinase activity has also been linked to the dismantling of the GTFs at promoters, once Pol II has left the promoter (Liu et al., 2004).

In addition to the GTFs, an additional multi-protein complex, mediator was found to be essential for the ability of transcriptional activators to stimulate high-level transcription by mediating interactions with the core transcription machinery (Cantin et al., 2003, Baek et al., 2006). The core mediator complex consists of 20-30 proteins, can exist in several distinct complexes and is conserved amongst humans and yeast (Taatjes, 2010). Multiple interactions of mediator subunits with Pol II and the GTFs, TFIID, TFIIE and TFIIIB function to enhance PIC formation and transcription (Mittler et al., 2001, Johnson et al., 2002, Baek et al., 2006, Soutourina et al., 2011, Cantin et al., 2003). Since mediator complexes can be as large as 1.2MDa, it is thought to provide an additional scaffold for transcriptional regulators to bind (Chadick and Asturias, 2005). For example the hepatocyte nuclear factor 4 (HNF4) protein is recruited directly to promoters by the mediator complex, stimulating transcription post-Pol II recruitment (Malik et al., 2002). Sub-mediator complexes containing the CTD-kinase CDK8, Cyclin C, MED13 and MED14 have also been detected, and the presence of the kinase module appears to correlate with a negative function of mediator in transcriptional regulation (see section 1.1.3.2) (Taatjes, 2010). Following promoter clearance, the mediator complex remains at the promoter possibly to facilitate the recruitment of other Pol II molecules (Svejstrup et al., 1997).

### ***1.1.2. Regulation of chromatin structure during transcription***

Epigenetics describes heritable changes to DNA that does not involve the alteration of the actual DNA sequence e.g. numerous histone modifications and direct DNA methylation can

dictate and regulate the transcriptional state of a gene. Due to the large amount of DNA per eukaryotic cell, it has to be highly condensed. This involves the wrapping of DNA around 4 different heterodimer complexes made up of core histone proteins (2x H2A and H2B heterodimers and (2x H3 and H4 heterodimers) to form nucleosomes (Wang, 1982). Compacting DNA through tight associations between histones and DNA however, reduces access of the transcription machinery to gene promoter sequences and the presence of nucleosomes at promoters is generally associated with the repression of transcription (Grunstein, 1990). Although the nucleosome structure is quite compact, the 8 highly-conserved N-terminal histone tails protrude out from the nucleosome core. Each histone tail contains multiple amino acids targeted for covalent post-translational modification by enzymes that establish 'active' or 'repressive' marks. These modifications form the basis for a histone code, where the 'marks' are 'read' and result in a specific biological outcome and where one histone modification may promote or exclude the establishment of another, in a process described as 'cross-talk'.

The major histone tail modifications involved in transcriptional regulation are acetylation, methylation, ubiquitination and phosphorylation. The presence of these marks can affect chromatin structure in two different ways. Firstly, tagging a histone tail with a particular modification can directly contribute to a conformational change in histone and nucleosome structure by affecting the net charge on the histone tail (Kouzarides, 2007). Secondly these modifications can provide binding sites for specialised complexes to compact or remove histones, thus regulating promoter access for the general transcription factors and Pol II.

#### **1.1.2.1. Histone acetylation**

Histone acetylation is usually associated with regions of active transcription. Acetylation occurs on lysine residues in both histone and non-histone proteins (Kim et al., 2006). It occurs primarily at the 5' ends and promoters of genes, although it has been detected throughout gene bodies (Wang et al., 2008b). Enzymes responsible for histone acetylation are known as histone acetyltransferases (HATs) and catalyse the transfer of acetyl groups from acetyl-CoA onto the lysine residues of histone tails. Individual HATs demonstrate poor catalytic activity, however

most are found in multi-subunit complexes that regulate HAT activity and proximity. There are currently 32 lysine residues within a single nucleosome, known to be differentially acetylated by different HAT complexes (For review see Choi and Howe, 2009).

There are 3 different HAT families found in mammals; the Gcn5-related N-acetyltransferases (GNAT), MYST and CBP/p300 families (Sterner and Berger, 2000). Each family preferentially acetylate different histone tail residues. For example GNAT members PCAF and GCN5 preferentially acetylate histone H3 at residues K9, K14 and K18 (Kouzarides, 2007). Whereas CBP/p300 can acetylate a much broader range of targets both *in vitro* and *in vivo*, which include histones 2A (K5), 2B (K12 and K15), H3 (K14 and K18) and H4 (K5 and K8) (Sterner and Berger, 2000, Kouzarides, 2007). In addition, CBP/p300, GCN5 and PCAF all contain bromodomains, a recognition domain consisting of 4 $\alpha$  helices and two hydrophobic loops that mediates binding to acetylated lysine residues (Haynes et al., 1992). In addition to histones, HATs can also acetylate various proteins associated with transcription. These include the general transcription factors TFIIE and TFIIIF (Imhof et al., 1997), elongation factor CDK9 (Fu et al., 2007) and p53 (Lill et al., 1997). The acetylation of p53 by CBP/p300 is essential for p53 stability and transcriptional regulation (Ito et al., 2001, Luo et al., 2004, Barlev et al., 2001).

The histone deacetylases (HDACs) counteract the action of HATs by removing the acetyl groups from lysine residues. This contributes to the compaction of DNA and transcriptional repression (De Ruijter et al., 2003, Wade, 2001). There are three families of HDACs; Class I, Class II and the SIR2 family (Kouzarides, 2007). Like the HATs, the HDACs have poor catalytic function without additional factors. HDACs are known to associate with at least 5 repressive complexes; SMRT (silencing mediator for retinoic acid and thyroid hormone receptors); NCoR (nuclear receptor co-repressor); NuRD (nucleosome remodelling and deacetylating); Co-REST (co-repressor of RE1 silencing transcription factor) and Sin3 (Jones et al., 2001, De Ruijter et al., 2003, Fischle et al., 2002, Ahringer, 2000, Belyaev et al., 2004). Interestingly both HATs and HDACs have been identified in the same complexes. All of the Class I HDACs (HDAC1, 2, 3 and 8) can interact with GCN5 and HDAC1 can additionally interact with the other GNAT family member PCAF (Yamagoe et al., 2003). However, neither HAT nor HDAC catalytic activity was investigated in these complexes. Further studies have demonstrated interactions between

GCN5 and the CLR3 HDAC complex in yeast to regulate H3K14 acetylation (Johnsson et al., 2009, Johnsson and Wright, 2010).

### **1.1.2.2. Histone methylation**

Histones can be mono or di methylated on arginine residues and mono, di or tri methylated on lysine residues by histone methyltransferases. Unlike acetylation, histone methylation can have either positive or negative influences on transcriptional regulation. Methylation of histone H3 at K4, K36 or K79 is associated with gene activation. H3K4me3 peaks at the 5' ends of genes, where it recruits TFIID associated factor TAF3 (Vermeulen et al., 2007), chromatin remodellers NURF/ISWI (Wysocka et al., 2006) and pre-mRNA splicing machinery (Sims et al., 2007) to enhance transcription and RNA processing.

Methylation of histone H3 at K9, K27 or histone H4 at K20 are associated with gene repression. H3K9me3 by the histone methyltransferase SUV39H1/H2 directly recruits the HP1 (heterochromatin protein1) through its conserved chromodomain (Cheutin et al., 2003, Lachner et al., 2001). This facilitates chromatin compaction through the associations between HP1 and DNA methyltransferases such as DNMT1 (Smallwood et al., 2007). In addition H3K27me3, catalysed by the PRC2 (polycomb repressive complex) protein EZH2 is associated with the recruitment of PRC1 complexes and the DNA methyltransferases DNMT1, 3A and 3B (Vire et al., 2006). The overexpression of EZH2 is linked to several malignancies such as breast, colorectal, gastric and prostate cancers and is therefore now being investigated as a potential therapeutic treatment (Bracken et al., 2003, Varambally et al., 2002, Mimori et al., 2005, Watanabe et al., 2007, Albert and Helin, 2009).

In addition to lysine residues, arginine residues are also targeted for methylation. They can be mono, asymmetrically dimethylated (aDMA) and symmetrically dimethylated (sDMA) by different protein arginine methyltransferases (PRMTs) (Di Lorenzo and Bedford, 2010). Arginine demethylases have not been detected, instead residues are converted to citrulline by the peptidylarginine deiminases (Di Lorenzo and Bedford, 2010). Arginine histone methylation on H3R2me2a and H2AR3me2a has different transcriptional outputs. H3R2me2a by PRMT6, has been shown to prevent the methylation of H3K4 and the binding of effectors to inhibit

transcriptional activation (Iberg et al., 2008, Hyllus et al., 2007). Contrastingly H2AR3me2a is associated with active promoters and regulating histone acetylation (Li et al., 2010). Knockout studies of PRMT1 demonstrated a concomitant decline in H2AR3me2a and histone H3 and H4 acetylation, whilst K9 and K27 levels increased (Huang et al., 2005).

#### **1.1.2.3. Histone ubiquitination**

The ubiquitination of histones H2A (K119) and H2B (K20) exhibit contrasting effects on gene transcription. Histone H2AK119Ub is associated with gene repression. H3K27me3 facilitates the recruitment of the PRC1 complex which contains the E3-ubiquitin ligase RING2/Bmi1 (Francis et al., 2001). RING2/Bmi1 can mono-ubiquitinate H2AK119, causing the recruitment of DNA methyltransferases leading to gene silencing (Wang et al., 2004, Wei et al., 2006, Wu et al., 2008, Vire et al., 2006). Conversely, H2BK20Ub is associated with highly transcribed genes (Minsky et al., 2008). Unlike H2A ubiquitination, H2BK20Ub is conserved in yeast (Kouzarides, 2007). This modification is catalysed by both RNF20/RNF40 and UbcH6 in humans and by Rad6/Bre1 in yeast (Kouzarides, 2007). H2BK20Ub has been shown to be a required for the trimethylation of H3K4, although this may not be the only pre-requisite (Foster and Downs, 2009, Sun and Allis, 2002). Furthermore, H2BK20Ub has also been shown to stimulate elongation through interactions with the histone chaperone FACT (Pavri et al., 2006).

#### **1.1.2.4. Histone phosphorylation**

The phosphorylation of histone H3 at serine 10 correlates with the activation of transcription. Through the induction of signalling pathways, H3S10 was shown to be transiently phosphorylated at several gene loci including the c-Jun and c-Fos genes, resulting in their transcriptional activation (Mahadevan et al., 1991). Enzymes that have been shown to phosphorylate H3S10 are the mitogen and stress response kinases MSK1 and 2 and the NFkB pathway activator IKKα (Yamamoto et al., 2003, Soloaga et al., 2003). This modification can be removed by several phosphatases, most notably by PP2A (Nowak et al., 2003). Interestingly, the H3 region surrounding serine 10 also contains lysine residues 9 and 14 that can be inhibitory or stimulatory for transcription, depending on their acetylation or methylation status

(Nowak and Corces, 2004). The phosphorylation of serine 10 has been shown to stimulate the acetylation of lysine 14, through the association with the GCN5 HAT complex (Lo et al., 2000, Clayton et al., 2000). However, this is not a universal trend, as demonstrated by the induction of the c-Jun gene, where the use of kinase inhibitors did not markedly affect histone acetylation (Brami-Cherrier et al., 2007, Thomson et al., 2001). In addition, the phosphorylation of serine 10 does not affect H3K9me, although it may prevent interactions between HP-1 and H3K9me, therefore maintaining an open chromatin state (Fischle et al., 2005).

#### **1.1.2.5. *ATP-dependent chromatin remodellers***

Promoter access by general transcription factors often requires the remodelling of chromatin structure to release the association of DNA with histones and/or positionally shift nucleosomes to expose promoter sequences. Using energy derived from ATP hydrolysis, nucleosomes are remodelled by 4 different families of ATP-dependent chromatin remodellers. These are the SWI/SNF, ISWI, CHD and INO80 families (Eberharter and Becker, 2004). Each family is associated with different aspects of chromatin remodelling and can be recruited through transcriptional activators or through individual domains. For example the SWI/SNF remodellers contain a bromodomain (Reviewed in Liu et al., 2011) and the ISWI family contain a SANT and SLIDE domain for direct nucleosome and DNA binding (Boyer et al., 2004). Recognition of acetylated lysine residues by the bromodomain of SWI/SNF complexes can facilitate the recruitment of remodelling complexes to the promoters of genes that have been targeted for activation by prior lysine acetylation mediated by HATs. Both the SWI/SNF and CHD family can slide and eject nucleosomes to augment transcription (Brown et al., 1996, Clapier and Cairns, 2009, Tran et al., 2000). Furthermore, SWI/SNF has also been shown to directly displace H2A/H2B nucleosomal dimers (Bruno et al., 2003). Besides nucleosomal sliding, the ISWI family are involved in nucleosome re-assembly and therefore also have a negative role in histone remodelling (Fazzio and Tsukiyama, 2003).



### **1.1.3. The CTD kinases and regulation of the CTD**

Transcription of Pol II-dependent genes is dependent on a repeated heptapeptide sequence (YSPTSPS), contained within the C-terminal domain (CTD) of the largest subunit of Pol II, Rpb1. The CTD is conserved in eukaryotes, however there is variation within the number of heptapeptide repeats; generally yeast have 26 repeats whereas mammals contain 52 (Palancade and Bensaude, 2003). Sequential deletion of CTD repeats in yeast demonstrated that less than 10 repeats were lethal and 13 repeats were established as the minimal requirement for viability (Nonet et al., 1987). Replacement of serine residues with alanine or glutamic acid residues was also shown to be lethal (West and Corden, 1995). Serine residues 2, 5 and 7 are targeted for phosphorylation by the cyclin-dependent CTD kinases CDK7, CDK8 and CDK9, allowing the recruitment of initiation, elongation and processing factors to bind, interact and regulate the transcriptional machinery (For a review see Buratowski, 2009)

Pol II is recruited to promoters with a poorly phosphorylated or hypophosphorylated CTD to form the PIC (Lu et al., 1991). Once recruited, the CTD is hyperphosphorylated, predominantly on serine 5 residues by the TFIIH subunit CDK7 and/or CDK8 (Komarnitsky et al., 2000, Lu et al., 1992, Hengartner et al., 1998). Hyperphosphorylation at serine 5 residues facilitate promoter escape and progression to elongation (Yamamoto et al., 2001). Immediately after promoter escape, capping enzymes are recruited through the serine 5 CTD motif to prevent degradation of nascent RNA (Rodriguez et al., 2000). Furthermore, capping enzyme activity can be stimulated *in vitro* by serine 5 phosphorylation (Wen and Shatkin, 1999). In addition, the CTD interacts with the splicing machinery and is required for efficient splicing (Misteli and Spector, 1999). In yeast, methyltransferases can bind phosphorylated serine 5 residues and stimulate transcription through the H3K4me3 modification (Hampsey and Reinberg, 2003). The serine 5 phosphorylation mark is gradually removed as elongation progresses through the binding and activation of CTD phosphatase Rtr1 (Mosley et al., 2009).

The gradual decrease in serine 5 phosphorylation, followed by the concomitant increase of serine 2 in the gene body and towards the 3' end of genes is a hallmark of an elongating Pol II complex (Saunders et al., 2006). Serine 2 phosphorylation is primarily catalysed by CDK9 (as

part of the positive elongation factor, pTEFb, complex) but can also be targeted by CDK8 (Marshall et al., 1996, Borggreffe et al., 2002). The use of two CDK9 kinase inhibitors, Flavopiridol and 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB), demonstrated the direct correlation between serine 2 phosphorylation and correct 3' end processing (Ni et al., 2004, Glover-Cutter et al., 2008). Furthermore, deletions in the CDK9 yeast homologue, Ctk1, also result in uncharacteristic 3' end processing through the incapability of recognising polyadenylation sites (Ahn et al., 2004).

#### **1.1.3.1. CDK7**

CDK7 and its partner Cyclin H are subunits of TFIIH (Roy et al., 1994, Fisher and Morgan, 1994). CDK7 and cyclin H can form a sub-complex with the RING-finger protein MAT1 forming the CDK-activating kinase (CAK) (Yee et al., 1995, Tassan et al., 1995a, Tassan et al., 1994). The presence of MAT1 in CAK enhances CDK7 kinase activity (Busso et al., 2000). CAK can regulate the cell cycle through the phosphorylation and regulation of other CDKs such as CDK1, 2, 4 and 6 (for a review see Lolli and Johnson, 2005). Interestingly, the affinity of CAK for the Pol II CTD is significantly lower than that of TFIIH (Rossignol et al., 1997).

There are two residues that contribute to the regulation of CDK7. Firstly, the phosphorylation on threonine 170 of the T-loop is required for CDK7 activity (Fisher et al., 1995, Devault et al., 1995). Secondly, the phosphorylation site at serine 164 is required for enhanced Cyclin H binding to CDK7 (Martinez et al., 1997). Another regulator of CDK7 and Cyclin H is the U1 snRNA (Kwek et al., 2002). *In vitro* kinase assays demonstrated that U1 snRNA, with Cyclin H, could significantly enhance CDK7 kinase activity (O'gorman et al., 2005). Whether U1 snRNA and MAT1 can functionally co-operative together to stimulate CDK7 kinase activity remains to be detected.

Until recently, the role of serine 7 residues on the Pol II CTD in transcription and processing was not well understood. Recent studies demonstrated that mutation of serine 7 to alanine in the CTD reduced transcription of small nuclear RNA (snRNA) genes by Pol II and were severely compromised in 3' end formation (Egloff et al., 2007). Interestingly, this mutation had no effect on protein-encoding genes (Chapman et al., 2007). Both the yeast homologue of CDK7, kin28,

and CDK7 can phosphorylate the CTD at serine 7 residues at both protein coding and snRNA genes (Glover-Cutter et al., 2009, Akhtar et al., 2009). Further information on the role of serine 7 phosphorylation was provided recently, when the first protein found to interact with this phosphorylated residue was described. The RNA Pol II-associated protein 2 (RPAP2), a phosphatase, was shown to bind to the serine 7 phospho-epitope and dephosphorylate serine 5 residues on snRNA genes (Egloff et al., 2012). By dephosphorylating serine 5 residues, RPAP2 is regulating the transition from transcriptional initiation to elongation.

### **1.1.3.2. CDK8**

CDK8 and its cyclin partner Cyclin C form complexes to primarily phosphorylate serine 5 and possibly serine 2 CTD residues (Rickert et al., 1999, Tassan et al., 1995b, Sun et al., 1998, Hengartner et al., 1998) and form part of mediator complexes. The mediator protein MED12 was recently shown to be required for CDK8 kinase activity (Knuesel et al., 2009b). CDK8 has been shown to stimulate transcription through CTD-independent mechanisms. CDK8 was shown to phosphorylate H3S10, leading to the acetylation H3K14 by the HAT GCN5 (Meyer et al., 2008). Subsequent CDK8 knockdown resulted in the global reduction of total histone H3 phosphorylation and acetylation (Meyer et al., 2008). Similarly, knockdown experiments demonstrated the requirement of CDK8 for p53-dependent activation of the p21 gene (Donner et al., 2007). Recently, CDK8-containing mediator complexes were shown to positively regulate the serum-response genes *FOS*, *EGR1*, 2 and 3 by augmenting the recruitment of CDK7 and CDK9 (Donner et al., 2010). Interestingly, both PIC formation, Pol II recruitment and histone acetylation were unchanged during CDK8 knockdown (Donner et al., 2010).

CDK8 can also associate with mediator complexes to negatively regulate transcription (Taatjes, 2010). There are several mechanisms whereby CDK8 can functionally inhibit transcription. Firstly, CDK8-mediator complexes can stimulate the repressive H3K9me2 chromatin mark through the recruitment of the G9a-histone methyltransferase (Ding et al., 2008). Secondly, the CDK8-subcomplex can bind to mediator and directly prevent Pol II promoter recruitment (Knuesel et al., 2009a). Similarly, the CDK8 yeast homologue Srb10, can directly phosphorylate the Pol II CTD prior to PIC formation and prevent Pol II promoter binding

(Hengartner et al., 1998). Finally, CDK8 can phosphorylate TFIIF *in vivo* to prevent both kinase activity and transcriptional activation (Akoulitchev et al., 2000).

### **1.1.3.3. CDK9**

The pTEFb (positive transcriptional elongation factor b) complex comprises the CDK9 kinase and its partner Cyclin T1. When Cyclin T1 is mutated, CDK9 has about 10% activity, therefore Cyclin T1 is a crucial cofactor for CDK9 function (Peng et al., 1998a). CDK9 preferentially binds Cyclin T1 but can also associate with Cyclin T2a, T2b and K (Peng et al., 1998b, Fu et al., 1999). pTEFb is able to regulate the transcription of many genes by phosphorylating the CTD and overcoming Pol II promoter-proximal pausing through phosphorylation of the pausing-associated factors NELF and DSIF (see section 1.1.5) (Marshall et al., 1996, Fujinaga et al., 2004, Kim and Sharp, 2001). In addition, the use of CDK9 inhibitors DRB and Flavopiridol highlighted the requirement for CDK9 to prevent the premature disassociation of Pol II from DNA and the production of truncated transcripts a process called abortive transcription (Marshall and Price, 1992, Marshall and Price, 1995, Marshall et al., 1996, Chao and Price, 2001, Chao et al., 2000). To prevent abortive transcription, pTEFb is the only CTD kinase capable of travelling with the elongation complex (Isel and Karn, 1999, Ping and Rana, 1999).

pTEFb activation and availability is highly regulated. In the nucleus, pTEFb complexes exist in a state of equilibrium. Approximately half of pTEFb is inactive and located in high molecular weight complexes in association with HEXIM (hexamethylene bis-acetamide inducible 1) proteins and 7SK snRNA (Yang et al., 2001, Nguyen et al., 2001). Cellular stresses such as heat shock, UV exposure and actinomycin D treatment facilitate the dissociation from the inactive complex (Biglione et al., 2007, Nguyen et al., 2001, Yang et al., 2001). CDK9 kinase inhibition by DRB or Flavopiridol also causes pTEFb to be released (Biglione et al., 2007).

CDK9 is also post-translationally regulated through phosphorylation and acetylation. The phosphorylation of threonine 186 is required for CDK9 activation (Wang et al., 2008a), by facilitating the binding of activators such as G-actin (Qi et al., 2011). However, this phosphorylated form of CDK9 also has an affinity for HEXIM-1 and inactive complexes (Chen et al., 2008). Threonine 186 is located within the T-loop, an evolutionarily conserved loop that

blocks access to the catalytic core of CDKs (Russo et al., 1996). Dephosphorylation of threonine 186 is a prerequisite for CDK9 release, as shown by the phosphatases PP2B and PP1a (Chen et al., 2008). Conversely, calcium signalling was shown to activate the kinase activity of CDK9 through phosphorylation of threonine 186 by the calcium/calmodulin-dependent kinase 1D (CaMK1D) (Ramakrishnan and Rice, 2011). Therefore threonine 186 phosphorylation is required for the formation of both active and inactive CDK9 complexes and may be regulated by the availability of other proteins such as HEXIM-1. The dephosphorylation of threonine 29 (Zhou et al., 2009) and serine 175 by protein-phosphatase 1 (Ammosova et al., 2011a) also appear to stimulate kinase activity.

CDK9 activity is also regulated by acetylation. The HAT p300, can acetylate CDK9 both *in vitro* and *in vivo* on its lysine 44 residue (Fu et al., 2007). This was shown to enhance the elongation of Pol II complexes (Fu et al., 2007). Subsequently, CDK9 is deacetylated by the NCoR complex containing HDAC1 and HDAC3 (Fu et al., 2007). The acetylation of lysine 48 by GCN5 however appears to inhibit the kinase activity of CDK9, possibly by interfering with  $Mg^{2+}$  or ATP binding (Sabo et al., 2008). The level of cross-talk or interplay between these modifications is yet to be established.

Due to its critical importance for transcriptional elongation, both cellular and viral activators can functionally recruit pTEFb to promoters to stimulate transcription. The main cellular recruiter of pTEFb is the chromatin association factor Brd4 (see section 1.1.4), where it can bind and directly sequester pTEFb from its inactive complex (Jang et al., 2005, Yang et al., 2005, Krueger et al., 2010). The induction of cellular stress through UV and Hexamethylene bisacetamide (HMB) treatment, results in the increased recruitment of pTEFb by Brd4 to inducible gene loci (Ai et al., 2011b). In addition, Brd4-dependent recruitment of pTEFb to the Cyclin D1, Cyclin D2, Orc2 and Mcm2 promoters increases the transition from G1 to S phase (Mochizuki et al., 2008). The proto-oncogene c-MYC can bind both subunits of pTEFb to stimulate the transcriptional elongation from the *cad* (carbamoyl-phosphate synthase) promoter (Gargano et al., 2007, Kanazawa et al., 2003, Eberhardy and Farnham, 2001). c-MYC can also recruit pTEFb to promoters to overcome promoter-proximal pausing (Rahl et al., 2010).

The pTEFb complex is also hijacked by viral activators to augment the transcription of viral genes. Most notably, the human immunodeficiency virus-1 (HIV-1) transcriptional regulator Tat can interact directly with Cyclin T1 (Herrmann and Rice, 1993). These interactions facilitate the recruitment of pTEFb to the viral long-terminal repeat (LTR) to stimulate viral protein synthesis (Isel and Karn, 1999, Kim et al., 2002). Moreover, Brd4 and the HIV-1 Tat protein can both functionally compete for pTEFb binding, relinquish pTEFb from its inactive complex and recruit it to promoters (Krueger et al., 2010, Yang et al., 2005, Bisgrove et al., 2007). Similarly, the human T-Lymphocyte virus type I (HTLV-1) protein Tax competes with Brd4 and HEXIM-1 for Cyclin T1 binding (Cho et al., 2007, Cho et al., 2010). In the absence of Brd4, pTEFb stimulation of Tax-dependent viral genes is greatly enhanced (Cho et al., 2007, Cho et al., 2010).

Both Cytomegalovirus (CMV) and the Herpes simplex virus 1 (HSV-1), two members of the *Herpesviridae* family, have also been shown to utilise pTEFb for their viral transcription. Initially, CDK9 was found to localize with IE1 and IE2 CMV viral proteins to facilitate intermediate-early gene expression (Tamrakar et al., 2005). Further studies demonstrated both viral and cellular factors including Cyclin T1, CDK7 and Brd4 were required (Kapasi and Spector, 2008, Kapasi et al., 2009). CDK9 can directly associate with the HSV-1 intermediate-early protein ICP22, to optimise viral replication by regulating the expression of late genes (Durand and Roizman, 2008, Durand et al., 2005). Interestingly, Kaposi's sarcoma-associated herpesvirus (KSHV) K-cyclin can directly interact with CDK9 to phosphorylate p53 however, whether these interactions have transcriptional consequence remains to be detected (Chang and Li, 2008).

CDK9 has also been shown to directly or indirectly regulate histone modification. CDK9 can directly phosphorylate histone H1, resulting in its dissociation from DNA during skeletal muscle differentiation (O'brien et al., 2010, O'brien et al., 2011). Knockdown of CDK9 also resulted in the complete loss of H2BK20Ub and subsequent H3K4me3 (Pirngruber et al., 2009). Whether CDK9 can augment the ubiquitination of H2BK20 by regulating an ubiquitin ligase or whether this is dependent on serine 2 CTD phosphorylation is unknown.

#### **1.1.4. Brd4**

Brd4, originally called mitotic chromosome-associated protein (MCAP), was identified in HeLa cells bound to chromatin and required for cell cycle progression into M phase (Dey et al., 2000). Brd4 belongs to the highly conserved BET family of proteins which contain two tandem bromodomains, a SEED domain and an extra-terminal domain (ET) and are found in mammals and yeast (Zeng and Zhou, 2002). Knockout studies of both yeast and mammalian BET family homologues including Brd4 are lethal, confirming their importance in cell survival (Houzelstein et al., 2002, Ladurner et al., 2003).

In normal cells, Brd4 is constitutively expressed and almost all is exclusively associated with chromatin (Ai et al., 2011b, Dey et al., 2000). Both Brd4 bromodomains, although low in homology with each other (Nakamura et al., 2007), consist of 4 $\alpha$  helices and two loops enabling Brd4 to bind di-acetylated histone tails on histones H3 (K9 and 14) and H4 (K5 and K12) (Dey et al., 2003). These interactions are weak and transient in nature and require additional proteins for prolonged stabilisation (Liu et al., 2008). This may be to counteract any non-specific binding of Brd4 to a ubiquitous histone tail marker.

One important role of Brd4 is the binding and recruitment of pTEFb to active promoters to facilitate transcription (Jang et al., 2005, Yang et al., 2005). Originally Brd4 was thought to interact with pTEFb through its bromodomains (Yang et al., 2005). However, Brd4 crystal structures and refined Brd4 mutants disproved this observation (Bisgrove et al., 2007, Vollmuth et al., 2009). Instead Brd4 contains a unique C terminal domain, absent amongst other human BET family members, capable of binding both pTEFb components (Bisgrove et al., 2007).

Like all BET family proteins, Brd4 remains bound to chromatin throughout mitosis, recruiting pTEFb prior to telophase to induce post-mitotic transcription (Yang et al., 2008, Dey et al., 2009). Subsequently, Brd4 knockdown causes cells to remain in G<sub>1</sub> arrest and undergo apoptosis (Mochizuki et al., 2008, Yang et al., 2008). Further analysis revealed 1108 genes that were downregulated in the absence of Brd4, including the E2F family of transcription factors and c-MYC, both of which are required for S phase transition (Mochizuki et al., 2008). Reintroduction of Brd4 rescued cells from G<sub>1</sub> and enabled them to enter S phase as normal

(Mochizuki et al., 2008). Brd4 also interacts with the key S phase protein complex replication factor C (RPC) both *in vitro* and *in vivo* (Maruyama et al., 2002). Brd4 over-expression and binding to RPC inhibits S phase progression, indicating that Brd4 expression needs to be highly regulated (Maruyama et al., 2002). Therefore Brd4 is required to recruit pTEFb to promoters to augment the transcription of genes required to regulate the cell cycle.

The SEED domain is characterised by poly-serine residues scattered between both glutamic acid and aspartic acid residues (Florence and Faller, 2001). The relevance of the SEED domain is yet to be determined unlike the ET domain. Recently Rahman *et al* discovered the ET domain interacted with several multi-protein complexes NSD3, JMJD6, CHD4, ATAD5 and GLTSCR1 (Rahman et al., 2011). Interestingly NSD3 and CHD4 are both associated with conflicting epigenetic markers, implying that Brd4 can both positively and negatively regulate gene transcription. NSD3 is a histone methyltransferase which catalyses the tri-methylation of lysine 36 residues on histone H3, associated with transcriptional activation and elongation (Tompa and Madhani, 2007, Xiao et al., 2003). Conversely, CHD4 is a component of NuRD, and acts as a transcriptional repressor through its histone deacetylase activity (Xue et al., 1998). The other proteins that interact with the ET domain of Brd4 have not been properly studied. The association with both NSD3 and CHD4 suggest Brd4 can regulate transcription independently of pTEFb (Rahman et al., 2011).

As well as the involvement with the elongation stage of transcription, Brd4 has also been linked to transcriptional initiation. Interactions with NFκB through both bromodomains augmented the transcription of NFκB-inducible genes (Huang et al., 2009). Furthermore, known interactions with mediator complexes in the absence of CDK8 have been detected at promoters, although the significance of these interactions are yet to be elucidated (Wu et al., 2003b).

Apart from cellular transcription, Brd4 also plays a critical role in the life cycles of various viruses including Kaposi's sarcoma-associated herpesvirus (KHSV), human Papillomavirus (HPV) and EBV. Initially, Brd4 was found to facilitate viral genome maintenance of the Bovine Papillomavirus (BPV) through associations with the bovine E2 protein and host cell chromosomes during mitosis (You et al., 2004). Further analysis demonstrated that Brd4 could bind to many different E2 proteins found in varying strains of HPV, although viral segregation



was found to be a Brd4-independent process in these cells (McPhillips et al., 2006, Schweiger et al., 2006). Instead Brd4 was found to have a transcriptional regulatory function (Schweiger et al., 2006).

The transcription of the HPV oncogene proteins E6 and E7 is negatively regulated by E2 (Dong et al., 1994). Loss of E2 function can lead to the expression of E6 and E7 resulting in malignancy (Goodwin and Dimaio, 2000). To repress E6/E7 transcription, E2 was demonstrated to bind and sequester Brd4 in the absence of pTEFb to viral promoters (Wu et al., 2006). Further studies have shown that E2 functionally competes with pTEFb for Brd4 binding (Yan et al., 2010). Conversely, using shRNA to Brd4, Schweiger *et al* demonstrated that E6 and E7 viral transcription could not be activated (Schweiger et al., 2007). Therefore Brd4-dependent silencing of HPV genes could be strain specific or require additional cellular factors (Smith et al., 2010, Senechal et al., 2007). The Brd4-E2 interaction could also function to stabilise E2. The E2 proteins have a short half life and are rapidly turned over (Gagnon et al., 2009). Over-expression of Brd4, substantially increased the half life of E2 by protecting it from proteasome degradation (Gagnon et al., 2009). This was also verified using the BPV and HPV 18 E2 proteins, where interactions with the Brd4 C terminal domain alone were sufficient to prevent the poly-ubiquitination and degradation by the proteasome (Zheng et al., 2009).

Brd4 has also been shown to interact with other viral proteins such as the KHSV latency-associated nuclear antigen or LANA (Viejo-Borbolla et al., 2005). Like BPV E2, the colocalisation of LANA and the KHSV viral episome with Brd4 and host mitotic chromosomes have been detected (You et al., 2006). Whether Brd4 plays a critical role in KHSV viral tethering remains to be determined. To facilitate cellular proliferation, LANA also upregulates the CDK2 promoter and CDK2 expression (Wong et al., 2004). Transient Brd4 and LANA co-expression assays demonstrated the ability of Brd4 to repress the LANA-dependent activation of the CDK2 promoter (You et al., 2006). Interactions with the EBV latent protein EBNA 1 have also been detected (Lin et al., 2008). Interactions with EBNA 1 was shown to regulate EBNA 1-dependent transcriptional regulation and appeared not to play a part in EBV viral replication (Lin et al., 2008).

All BET proteins are variably spliced and expressed as either a long or short form. The Brd4 shorter form (HUNK-1) contains both bromodomains and the ET domain, but is missing the last 677 amino acids including the C terminal region (Wu and Chiang, 2007). Apart from being a potential dominant negative protein, there is no functional role for HUNK-1 even though it is readily expressed in many cell types (French et al., 2001). A t(15;19)(q13,p13.1) translocation fuses a testes specific protein called NUT to a truncated form of Brd4 (French et al., 2003, French et al., 2001). Interestingly the difference between this truncated form and HUNK-1 is 3 amino acids. This translocation causes an aggressive midline carcinoma which is often fatal within 3 months (Vargas et al., 2001). Until recently, the function of the Brd4-NUT fusion protein was unknown. Reynoird *et al* demonstrated the Brd4-NUT fusion protein to have both DNA and p300 binding activity (Reynoird et al., 2010). Surprisingly, aberrant hyperacetylation of histones by the Brd4-NUT-p300 complexes resulted in transcriptional repression (Reynoird et al., 2010). Also, the fusion protein could bind acetylated p53 (Reynoird et al., 2010). Further investigation is needed to determine the significance of p53 binding and whether transcriptional repression through acetylation is detected in other cell lines.

### **1.1.5. Transcriptional regulation through Pol II pausing**

Promoter-proximal pausing describes the accumulation of Pol II and associated factors after promoter escape and often occurs around 50 nucleotides downstream of the transcriptional start site. It was first described on the *Drosophila hsp70* gene, and involves two factors DRB Sensitivity Inducing Efactor (DSIF) and Negative Elongation Efactor NELF (Wu et al., 2003a). DSIF-NELF-mediated pausing has since been documented at many inducible genes including; *c-FOS* and *c-JNB* (Fujita et al., 2008); *TNF $\alpha$*  (Adelman et al., 2009) and *MKP-1* (Fujita et al., 2007). Recent genome-wide analysis has revealed Pol II promoter pausing, in association with DSIF and NELF occurs globally in *Drosophila* and Mouse embryonic stem cells (Gilchrist et al., 2010, Rahl et al., 2010). The exact reason why constitutively active genes are paused is unknown. However, the advantage of a paused Polymerase at inducible genes is the rapid induction of transcription. Therefore no time is lost by recruiting the general transcription factors, Pol II, elongation factors and histone modifiers. Additionally, pausing may permit the co-

ordinated capping and splicing of emerging nascent RNA, termed elongation checkpoint control (Pei et al., 2003, Glover-Cutter et al., 2008).

Transcription of paused genes can be induced in a stimulus-dependent manner that results in the specialised recruitment of pTEFb (Gomes et al., 2006, Fujita et al., 2009, Fujita et al., 2008, Fujita et al., 2007, Lis et al., 2000). Transcriptional pausing is relieved through pTEFb-dependent phosphorylation of NELF, DSIF and the serine 2 residues on the Pol II CTD (Fujinaga et al., 2004, Kim and Sharp, 2001, Ivanov et al., 2000, Isel and Karn, 1999, Tian et al., 2003). Further experiments have shown pausing at non-inducible promoters may prevent nucleosome formation (Gilchrist et al., 2010, Gilchrist et al., 2008). The prevention of nucleosome formation by an accumulation of Pol II could potentially induce transcription much more efficiently.

#### **1.1.5.1. NELF**

The NELF protein complex contains four subunits NELF A, NELF B, NELF C or NELF D and NELF E and is expressed in most cell types (Yamaguchi et al., 1999, Narita et al., 2003). The NELF proteins are however only conserved in vertebrates and insects (Narita et al., 2003). NELF A is encoded by the Wolf-Hirschhorn syndrome gene WHSC2 (Wright et al., 1999). Wolf-Hirschhorn syndrome is a disease characterised by growth defects including shortness of stature and mental retardation (Rauch et al., 2001). Evidence supporting a potential role in growth regulation was confirmed when NELF A was shown to bind both Pol II and DSIF simultaneously (Yamaguchi et al., 2002). NELF B or Cofactor of BRCA1 (COBRA1) was initially found to interact with BRCA1 (Ye et al., 2001) and downregulate *estrogen receptor  $\alpha$*  (*ER $\alpha$* ) dependent-genes in breast epithelial cells (Aiyar et al., 2004). Interestingly, there may be a possible correlation between aberrant NELF B expression and several different cancers. Low NELF B expression is associated with malignant breast cancers (Sun et al., 2008), whilst, over expression of NELF B is readily found in upper gastrointestinal adenocarcinomas (Mcchesney et al., 2006). Further studies using shRNA directed against NELF B found that 134 genes were differentially regulated, among them were several gene clusters associated with cancer

development (Aiyar et al., 2007). Whether this is limited to NELF B and breast carcinoma has yet to be determined.

The functions of NELF C and NELF D although unknown are likely to be similar since both proteins are translated from the same mRNA using a different translational initiation codon (Narita et al., 2003). In addition they are both found in distinct NELF complexes (Narita et al., 2003). NELF E contains a RNA recognition motif (RRM) which facilitates the binding of NELF E to RNA (Yamaguchi et al., 2002, Rao et al., 2006, Rao et al., 2008). Therefore NELF-mediated pausing may initially start by binding to DSIF and Pol II through the NELF A subunit. As Pol II escapes the promoter and begins transcribing the gene, nascent RNA emerges and binds to NELF E. This may cause several conformational changes, leaving Pol II paused and unable to transcribe. After phosphorylation by CDK9 (Fujinaga et al., 2004), the NELF complex dissociates from Pol II and transcription can resume.

Interestingly, there are several lines of evidence supporting a positive-transcriptional role for NELF. Microarray studies in *Drosophila Melanogaster* have showed NELF to be associated with almost 50% of the most highly expressed genes in its genome (Lee et al., 2008). Similarly, NELF knockdown experiments demonstrated the reduction of Pol II and the epigenetic marks associated with transcriptional activation that led to the formation of nucleosomes (Sun and Li, 2010, Gilchrist et al., 2008). Interestingly, ChIP coupled to next-generation sequencing (ChIP-seq) has shown NELF and DSIF to both occupy the same gene loci as Pol II at both actively transcribed and paused genes suggesting that they may be both general transcription factors instead of specialised elongation factors (Gilchrist et al., 2010, Rahl et al., 2010).

NELF has also been shown to facilitate 3' end processing, by inducing a pTEFb-independent pause at the 3' end of U2 snRNA and  $\beta$ -Actin genes (Egloff et al., 2009). This pause was required in order to regulate proper termination (Egloff et al., 2009). Additionally, Narita *et al* demonstrated an interaction between NELF and the nuclear cap binding complex (CBC) (Narita et al., 2007). Similarly, this was required for correct 3' end processing for the transcription of histone genes (Narita et al., 2007). There seems to be overwhelming evidence that NELF can induce pausing at promoter proximal and distal regions. Moreover, this pausing may have negative roles at inducible genes and a stimulatory role at constitutively active ones.

### **1.1.5.2. DSIF**

DRB Sensitivity Inducing Factor (DSIF) is a heterodimer of two ubiquitously expressed proteins Spt4 and Spt5 (Wada et al., 1998a). Both are homologues to the yeast transcription factors Spt4 and Spt5 (Hartzog et al., 1996, Swanson et al., 1991). Importantly, Spt5 is the only Pol II-associated elongation factor to be conserved in all 3 domains; Eukaryotes, Prokaryotes and Archaea (Werner, 2007). In addition, genome-wide Spt5 knockdown suggest a role in preventing senescence and apoptosis independently of the p53 pathway (Komori et al., 2009).

The well characterised bacterial transcription factor, NusG, is a homologue of Spt5 (Reay et al., 2004). Similarly to NusG, Spt5 mediates the binding of DSIF to Pol II to stimulate and repress transcription (Ivanov et al., 2000). Recent *Drosophila* and archaeal Pol II/DSIF crystal structures have revealed that through its KOW domains, Spt5 may interact with both non-template DNA and nascent RNA adjacent to the Pol II clamp region (Klein et al., 2011, Missra and Gilmour, 2010). By binding RNA or DNA, these interactions could sterically hinder the elongating Pol II and be required to establish pausing with NELF. To overcome pausing, Spt5 is phosphorylated by pTEFb (Kim and Sharp, 2001) and unlike NELF, it remains bound to the elongating Pol II complex (Zhang et al., 2004), possibly to prevent further downstream pausing events and promote competent elongation (Cheng and Price, 2007, Zhu et al., 2007).

In addition to enhancing elongation and pausing, DSIF may also have a role in the capping of nascent mRNA. Capping of eukaryotic mRNA is carried out by the human capping enzyme (HCE) and the RNA 7-methyltransferase, which are required for preventing the degradation of nascent mRNA transcripts by ribonucleases (Furuichi and Shatkin, 2000). Spt5 has been shown to bind and stimulate the HCE, possibly by stabilising the weak interactions detected between the HCE and Pol II CTD (Wen and Shatkin, 1999). In addition Lindstrom *et al* documented that DSIF can interact with transcription factors TFIIS and TFIIIE, nucleosome re-modellers Spt6 and FACT (see section 1.1.6) and play a role in pre-mRNA splicing (Lindstrom et al., 2003). TFIIS is an elongation factor, implicated in the positive regulation of Pol II pausing by stimulating nascent RNA cleavage and preventing promoter-distal pausing (Izban and Luse, 1993, Izban and Luse, 1992). Therefore it appears Spt5 may also be regulating the activity of histone

chaperones throughout elongation. However, further investigation is required to determine the functional significance of these interactions.

DSIF has also been implicated in the regulation of the HIV-1 viral LTR. Initially DSIF was found to be a cellular factor required for Tat activation (Wu-Baer et al., 1998). Further studies showed that the over-expression of Spt5 also contributed to Tat-specific activation of the LTR (Kim et al., 1999). Spt5 knockdown resulted in the downregulation of viral transcription (Ping et al., 2004). Similarly to NusG, Spt5 maybe required in late elongation to prevent premature termination of both viral and cellular transcripts (Sullivan and Gottesman, 1992, Linn and Greenblatt, 1992).

### **1.1.6. Elongating through chromatin structure**

Following initiation and promoter clearance, in order to efficiently transcribe the length of the gene transcription unit, the elongating Pol II must overcome the obstacle of chromatinized DNA. To aid elongation through nucleosomal templates, histone chaperones are required to temporarily remove nucleosomes and promote their reassembly after Pol II passage. Two histone chaperones; Facilitates chromatin transcription (FACT) and Spt6 have been implicated in removing histones to stimulate transcription elongation. FACT is a heterodimer consisting of the Spt16 and SSRP1 proteins; it is recruited to elongating complexes through associations with Pol II (Mason and Struhl, 2003), DSIF (Orphanides et al., 1998, Lindstrom et al., 2003) and the ATP-dependent chromatin remodeller CHD1 (Kelley et al., 1999). FACT can interact directly with histone H2A/H2B and H3/H4 dimers and can physically mediate the removal of one H2A/H2B dimer (Orphanides et al., 1999, Belotserkovskaya et al., 2003).

Spt6 is a histone chaperone that interacts with histone H3 and the CTD of Pol II through its phosphorylated serine 2 residues (Endoh et al., 2004, Yoh et al., 2007, Bortvin and Winston, 1996). Spt6 can reassemble nucleosomes *in vitro* and is required for competent elongation in yeast (Bortvin and Winston, 1996, Hartzog et al., 1998). Using Spt6 mutants, Kaplan *et al* demonstrated that transcription could initiate from another initiation site located within the *FLO8* gene (Kaplan et al., 2003). This coincided with a notable absence of histone H4 within the gene (Kaplan et al., 2003). Therefore by reassembling histones after transcription, inappropriate

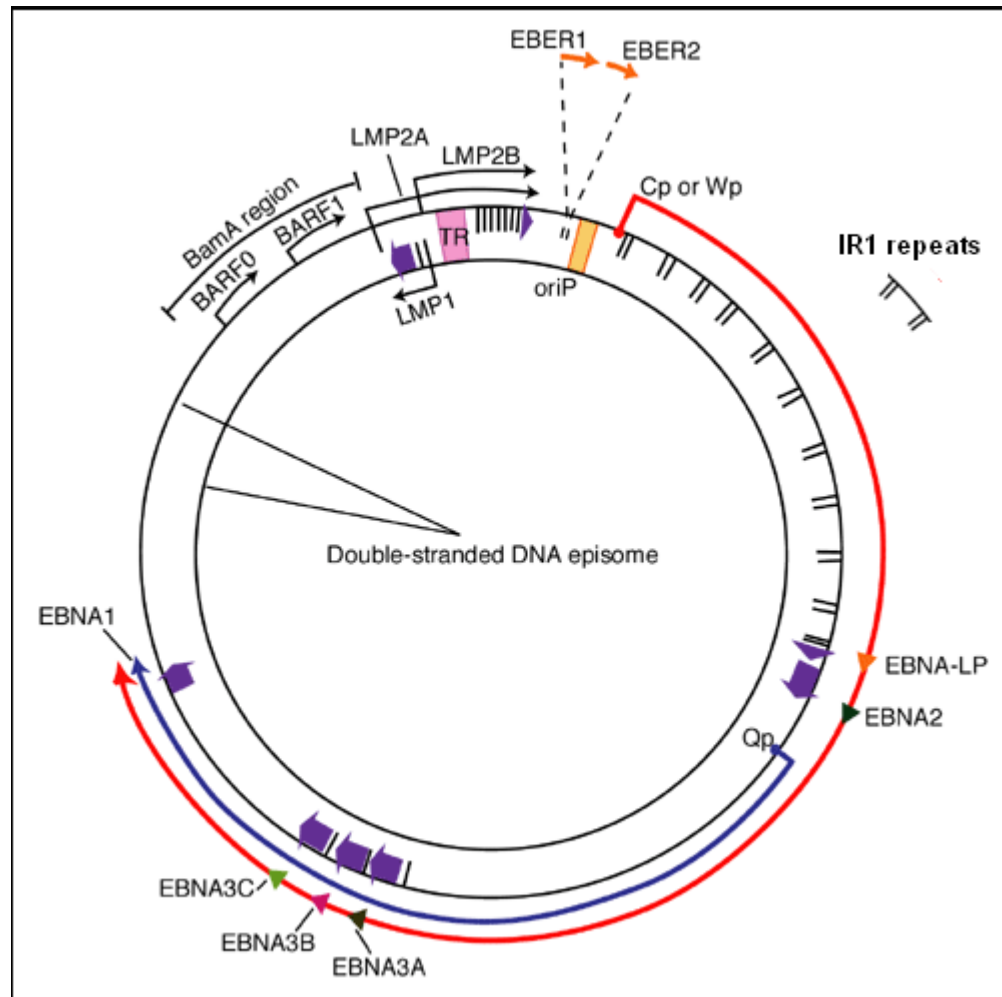
initiation and chromatin integrity is maintained to facilitate repeated rounds of transcription (Ardehali et al., 2009).

## 1.2. Epstein-Barr Virus

Epstein-Barr Virus (EBV) was initially discovered when viral-like particles were observed under the electron microscope in a series of Burkitt's Lymphoma cell lines (Epstein et al., 1964). EBV belongs to the *herpesviridae* family of viruses, which are double-stranded viruses, capable of establishing both a replicative lytic and a dormant latent phase of infection. Herpesviruses are further sub-divided into either  $\alpha$ ,  $\beta$  and  $\gamma$  sub families. EBV is classified as a  $\gamma$ -herpesvirus, due to a limited B cell tropism and its ability to replicate in lymphoblastoid and epithelial cell lines *in vitro* (Roizman et al., 1981). EBV infection is spread by salivary contact and usually occurs asymptomatically during childhood. However, if infection is delayed until adolescence, infectious mononucleosis (IM) can occur in 50% of cases (Henke et al., 1973). Upon initial infection EBV establishes a lytic infection, possibly through the epithelial cells of the upper respiratory tract. After replicating, EBV penetrates the B cell pool by interacting with the B cell-specific receptor complement receptor 2 (CD21) (Fingerroth et al., 1984). Once internalised, EBV establishes a lifelong persistence through its four latency transcription patterns (latency 0, I, II or III).

### 1.2.1. EBV infection in vitro

EBV can immortalise resting B cells *in vitro* to establish permanently proliferating lymphoblastoid cell-lines (LCLs) (Henle et al., 1967). It is estimated that EBV can transform over 50% of all resting B cells in the absence of a T cell-mediated immune response (Thorley-Lawson and Allday, 2008). The importance of a fully functioning T cell response was demonstrated through the use of the immunosuppressive drug cyclosporin A. Bird *et al* demonstrated Cyclosporin A treatment correlated with the outgrowth of EBV positive B cells from EBV positive donors (Bird et al., 1981). EBV-immortalised LCLs express all latent proteins; the 6 EBV Nuclear Antigens (EBNA 1, 2, 3A, 3B, 3C and LP); 3 Latent Membrane Proteins (LMP 1, 2A and 2B); 2 Epstein-Barr encoded RNAs (EBER 1 and EBER 2) and the BamH1 A



**Figure 1 The EBV genome.** EBV expresses 9 latent proteins, indicated by the purple arrows. The direction in which they are transcribed is also indicated. The red arrow indicates the polycistronic transcript deriving from Cp or Wp, including the variable IR1 repeats (10 shown) associated with a latency III phenotype. The inner blue arrow shows transcripts deriving from Qp, where only EBNA 1 transcripts are made in a latency I phenotype. Adapted from (Young and Murray, 2003)



rightward transcripts (BART transcripts and miRNAs) known as latency III (see Figure 1).

Latency III is associated with B cell growth and proliferation.

### **1.2.2. EBV infection *in vivo***

Upon initial infection, the linear, dsDNA genome is able to circularise as an episome activating the latency III growth programme causing naïve B cells to proliferate (Adams and Lindahl, 1975). Unlike *in vitro*, these proliferating B cells do not persist because many of the proteins expressed are highly immunogenic and induce a strong cytotoxic T lymphocyte response (Rickinson and Moss, 1997). Infected B cells are thought to migrate to follicles to undergo the germinal centre (GC) reaction where they proliferate and differentiate into memory B cells or plasma cells (For review see Klein and Dalla-Favera, 2008). During the GC reaction, the latency III growth programme is downregulated to a latency II transcriptional programme which expresses EBNA 1, LMP 1, LMP 2A and LMP 2B only (Thorley-Lawson, 2001).

Although all EBV latency types have been detected in GC, the proteins expressed in latency II are thought to drive the survival of aberrant GC B cells that would normally undergo apoptosis (Spender and Inman, 2011, Roughan and Thorley-Lawson, 2009, Roughan et al., 2010, Babcock et al., 2000). For example B cells with a faulty B cell receptor (BCR) would normally undergo apoptosis due to its critical roles in signalling and proliferation (Tsubata et al., 1993). Importantly, EBV can provide survival signals to these non-functional cells (Caldwell et al., 1998, Caldwell et al., 2000, Bechtel et al., 2005). After the GC reaction, EBV establishes a lifelong persistence in memory B cells by expressing either EBNA 1 only (latency I) or no latent genes at all (latency 0) (Babcock et al., 1998). The mechanism of EBV latency-type switching is largely unknown. However, both cellular and viral epigenetic regulation of the EBV latent promoters is involved.

### **1.2.3. The EBV latent promoters**

#### **1.2.3.1. Wp**

Upon initial infection, the first viral transcripts detected arise from the W promoter (Wp) (Woisetschlaeger et al., 1990). Wp resides within the tandem IR1 repeats, which vary in number between viral isolates (see Figure 1). Transcripts initiated from each IR1 repeat contain a short 5' W<sub>0</sub> exon, followed by differential splicing of two repeated exons W<sub>1</sub> and W<sub>2</sub> (Speck et al., 1986). Therefore each viral episome contains multiple active W promoters. Different analysis of the IR1 repeat region has demonstrated a varying number of repeats including both 6 and 8.6 amongst individual cell lines (Allan and Rowe, 1989). Recent *ex vivo* studies showed a mean of 5 to 8 IR1 repeats present in IM patients (Tierney et al., 2011). Due to the lack of latent proteins in the viral capsid, Wp is regulated mainly by cellular factors.

Initially Sugano *et al* identified the B cell-specific regulator NF-κB as the first cellular factor found to bind and activate Wp (Sugano et al., 1997). However, other labs have been unable to validate this observation (Bell et al., 1998). Using Wp band shift assays, *Bell et al* identified 3 upstream activating sequences (UAS1-3), UAS1 was important for B cell specific activation and UAS2 and UAS3 were important for Wp activation in other cell lines (Bell et al., 1998). Further experiments revealed the ubiquitously expressed transcription factor YY1 not only bound to the UAS2, but was required for Wp activation in non-B cell lines such as T cells (Bell et al., 1998). Finer mapping of UAS1 revealed 4 binding sites (A-D), where A associated with the cAMP dependent CREB/ATF factors, B and D bound the B cell specific transcriptional activator Pax5 and region C interacted with the RFX family of regulators (Tierney et al., 2000a, Kirby et al., 2000, Bell et al., 1998). All were capable of binding UAS1 simultaneously and were required for Wp activity *in vitro* (Tierney et al., 2000a, Kirby et al., 2000). *In vivo* ChIP assays revealed Pax5 bound UAS1 in two LCLs and was required for immortalisation (Tierney et al., 2007).

Transcription from Wp results in the synthesis of detectable EBNA-LP and EBNA 2 proteins between 8 and 12 hours post transfection (Allday et al., 1989, Alfieri et al., 1991). After 3-6 days post infection, Wp activity is significantly reduced as the C promoter (Cp), located just upstream

of the first IR1 repeat is active (Woisetschlaeger et al., 1990). Following a timed B95.8 infection of primary B cells, Wp was methylated on CpG sites from day 7 onwards and >90% of Wp was methylated by day 18 (Tierney et al., 2000b). Further experiments showed Wp to be methylated at 13 CpG sites in LCLs and IM patients, some of which prevented associations with cellular transcription factors (Tierney et al., 2000b). Other experiments confirm that Wp methylation correlates with a decline in Wp activity, although the extent of methylation varies amongst different cell lines (Jansson et al., 1992, Minarovits et al., 1991, Elliott et al., 2004, Park et al., 2007).

### **1.2.3.2. Cp**

The viral C promoter (Cp), only active in B cells drives transcription of a ~120 kb pre-mRNA that is differentially spliced to generate messages encoding all the other EBNAs required for immortalisation and expressed in latency III (Contreras-Brodin et al., 1996, Bodescot et al., 1987, Bodescot and Perricaudet, 1987). Interestingly, Cp mutant or deleted viruses can still immortalise B cells through Wp usage (Swaminathan et al., 1993, King et al., 1980). Primary infections using an EBNA 2-deleted virus primarily utilised Wp and failed to switch to Cp usage, indicating that EBNA 2 is the main regulator of Cp (Woisetschlaeger et al., 1991). EBNA 1 can also enhance Cp transcription by binding the latent replication origin site, OriP (Puglielli et al., 1996, Schlager et al., 1996, Yates et al., 1985, Sugden and Warren, 1989).

Cp activity requires several cellular transcriptional factors. Most notably are the upstream CBF1 (RBP-Jk) and CBF2 binding sites that are required for EBNA 2-dependent Cp activation (Ling et al., 1993, Fuentes-Panana and Ling, 1998). NF-Y, SP1, SP3 and C/EBP transcriptional activators were also shown to interact with upstream elements of Cp in Rael BL cells (Nilsson et al., 2001). Further studies showed NF-Y and Sp1 were essential for Cp activation in the presence of EBNA 2, which may play an important role in the Wp/Cp switch (Borestrom et al., 2003). Interestingly, the binding of the chromatin boundary factor CTCF between OriP and Cp may contribute to the maintenance of specific latency (Chau et al., 2006). Chromosomal conformational capture (3C) techniques revealed that the binding of CTCF upstream of Cp and Wp in conjunction with both cellular and viral factors influenced the different tertiary chromatin

structures associated with latency I and latency III cells (Tempera et al., 2010, Tempera et al., 2011). Therefore CTCF may cause DNA looping, restricting the access of transcriptional machinery to various latent promoters (Tempera et al., 2010, Tempera et al., 2011).

Cp is also subject to epigenetic regulation. In latency III cells such as LCLs and some IM positive patients, no methylation of CpG sites is detected (Tierney et al., 2000b). Consistent with Cp activity, Cp was acetylated on histones H3 and H4 in latency III cells (Fejer et al., 2008, Alazard et al., 2003). However, as expected, in cells displaying latency I or II gene expression patterns, Cp is methylated on some or all of the 9 CpG dinucleotide sequences resulting in the inability to bind transcription factors and thus promoter inactivity (Bakos et al., 2007, Tierney et al., 2000b)

### **1.2.3.3. LMP 1p**

The LMP1 gene is entirely contained within the LMP 2A gene locus on the opposite DNA strand (see Figure 1); therefore transcription occurs in the reverse orientation. LMP1 transcripts are derived from 2 different latent promoters, depending on latency and cell type. This is reflected by the numerous transcriptional regulatory sites found upstream of the LMP1p termed the LMP1 regulatory sequence (LRS). EBNA 2 is known to regulate the proximal LMP1p, ED-L1 during latency III (Wang et al., 1990b). This promoter is bi-directional and EBNA 2 also activates transcription of the rightward LMP2B gene. Key regulatory regions required for EBNA 2-dependent transcription have been mapped within the LRS to include 2 RBP-Jk sites, a PU.1 binding site, a cAMP response element (CRE) and an AP-2 consensus site (Johannsen et al., 1995, Laux et al., 1994a, Jansson et al., 2007, Sjoblom et al., 1998). All of these sites play a role in EBNA-2 dependent ED-L1 activation. In addition, ED-L1 can be co-activated in conjunction with EBNA 3C through the PU.1 cognate sequence (Lin et al., 2002, Zhao and Sample, 2000).

In latency II, ED-L1 can additionally be activated by cellular factors. Sjoblom *et al* demonstrated a heterodimer complex of ATF-1 and CREB-1 could form at CRE and activate ED-L1 in the absence of EBNA 2 (Sjoblom et al., 1998). In addition, an upstream E-box motif, can regulate ED-L1 activity. The binding of upstream stimulatory factor 1 (USF1) to the E-Box

motif and over-expression of USF1 and USF2A enhanced ED-L1 expression in reporter assays (Sjoblom-Hallen et al., 1999). Conversely, co-repressor complexes containing mSin3A and histone deacetylases could also interact and with and repress transcription from the E-box.

A TATA-less, alternate LMP1p called LT-R1 (or ED-L1E) is activated exclusively in epithelial cells (Sadler and Raab-Traub, 1995). The transcript derives from within the terminal repeat region (TR) and transcripts are larger than ones detected in B cells from ED-L1 (Sadler and Raab-Traub, 1995, Gilligan et al., 1990). LT-R1 is regulated by an upstream GC box, located between -48 and -33 base pairs from the transcriptional start site (Tsai et al., 1999). Further experiments demonstrated that both Sp1 and Sp3 transcription factors interacted with the GC box and were required for its activity (Tsai et al., 1999). Experiments using HeLa cells infected with the EBV B95.8 virus in conjunction with STAT3 over-expression, upregulated LMP1 expression through the LT-R1 (Chen et al., 2003). Whether STAT3 can activate the LMP1 in a natural EBV host cell environment is yet to be determined. Similarly to Wp and Cp, LMP1p is silenced by CpG methylation in BL and some undifferentiated nasopharyngeal carcinoma (UNPC) cell lines (Masucci et al., 1989, Gregory et al., 1990, Hu et al., 1991).

#### **1.2.4. EBV- associated diseases**

##### **1.2.4.1. Burkitt's Lymphoma (BL)**

Burkitt's lymphoma (BL), first observed by Denis Burkitt (Burkitt, 1958) is classified as a monoclonal B cell tumour which can be sub-divided into 3 forms; endemic (Equatorial Africa), sporadic (Europe and The United States) or HIV/AIDS related. EBV was first implicated in the pathogenesis of endemic BL after its discovery in BL cell lines (Epstein et al., 1964). Consequently EBV was shown to be associated with nearly 100% of all endemic BL tumours derived from Equatorial Africa and New Guinea (Magrath, 1990, Pagano et al., 1973). However, the association of EBV in sporadic and HIV-related BL is less common, with approximately 15-25% and 30-40% incidence, respectively (Magrath, 1990). The age association of BL and the location of the tumour is also different in EBV<sup>+</sup> and EBV<sup>-</sup> BL tumours. EBV<sup>+</sup> BL tumours usually

occur in young children, located around the eye, jaw and abdomen; whereas EBV<sup>+</sup> BL tumours occur in the abdomen and associates with all age groups (Thorley-Lawson and Allday, 2008).

EBV-transformed LCLs and BL cells are phenotypically different; LCLs are larger and irregular in shape and mimic a phenotypically activated B lymphoblast (Gregory et al., 1987). Whereas BL cells express cell surface markers characteristic of a germinal centre B cell (Gregory et al., 1987). This is because the proliferation of BL cells is regulated by a c-MYC translocation, not EBV. Using conditionally-active EBNA 2 LCLs transfected with an active c-MYC gene, Polack *et al* demonstrated these cells could proliferate in a stimulus-independent manner to gain a growth phenotype similar to BL (Polack et al., 1996). In BL cells the translocation places the c-MYC gene (chromosome 8) under the control of a constitutively active heavy (chromosome 14) or light (chromosomes 2 or 22) chain immunoglobulin enhancer resulting in uncontrolled proliferation (Cesarman et al., 1987, Magrath, 1990, Bhatia et al., 1993).

The contribution of EBV to BL is still unknown. One possible role is to counteract the high apoptosis rate, a consequence of aberrant c-MYC expression (Milner et al., 1993, Gregory et al., 1991). However, in most cases of BL EBV establishes a latency I state, only expressing EBNA 1, which has no significant anti-apoptotic behaviour in BL (Kelly et al., 2006). Interestingly, approximately 15% of EBV<sup>+</sup> BL cell lines express a Wp-restricted latency, occurring because of an EBNA 2 gene deletion that results in the EBNA 3 family, EBNA-LP and EBNA 1 latent protein expression but not EBNA 2, LMP1, LMP2A or LMP2B since both Cp and the LMP gene locus are inactive (Kelly et al., 2002). These Wp-restricted BL cell lines are more protected from apoptosis than EBNA 1-only expressing BL cells (Kelly et al., 2006). Moreover, the Wp-restricted cells produce a Bcl-2 homologue (BHRF1), which counteracts apoptotic signals (Kelly et al., 2009).

The geographic regions associated with endemic BL also coincide with holoendemic malaria and decreases in BL incidence occur in areas where malaria eradication programmes are in place (Burkitt, 1983, Van Den Bosch, 2004). Furthermore, delayed-onset BL has been documented in immigrants moving to higher malaria incidence regions (Burkitt, 1983, Van Den Bosch, 2004). Therefore malaria is classed as an endemic BL co-factor, although its full role is

yet to be determined. Malaria has been shown to cause EBV reactivation and may contribute further by suppressing T-cell mediated responses (Donati et al., 2004, Ho et al., 1988b, Ho et al., 1986)

#### **1.2.4.2. *Hodgkin Lymphoma (HL)***

There are two different types of HL, classical HL and nodular lymphocyte predominant HL with only 1% of the tumour mass accounting for malignant cells in a surrounding microenvironment (Farrell and Jarrett, 2011). HL is a lymphoid malignancy associated with tumours of the spleen, liver, lymph nodes and bone marrow. Classical HL is characterised by the presence of Reed-Sternberg cells, often infected with EBV, which express high levels of EBV latent transcripts EBNA 1, LMP1, LMP 2A/2B, BART RNAs and the EBERs (latency II) (Weiss et al., 1989, Weiss et al., 1987, Fields et al., 2001).

The role of EBV in HL has not been fully elucidated, but there is mounting evidence that it promotes the survival of aberrant cells. Firstly, in EBV<sup>+</sup> HL, there is evidence of a monoclonal EBV genome suggesting infection occurred prior to malignancy (Gulley et al., 1994, Anagnostopoulos et al., 1989). Once activated naïve B cells undergo GC reactions, such as somatic hypermutation. Somatic hypermutation involves the introduction of mutations into immunoglobulin genes resulting in the optimised recognition of pathogens and immune response (Kuppers et al., 1993). Under normal conditions, if somatic hypermutation produces B cells that do not provide a selective advantage or are “crippled” in some way they undergo apoptosis (Rajewsky, 1996). Approximately 25% of HL cells contain “crippled” Ig genes, all of which are EBV<sup>+</sup>, therefore EBV may promote the survival of these aberrant B cells (Kanzler et al., 1996, Mancao and Hammerschmidt, 2007, Mancao et al., 2005).

Classical HL is also characterised by the loss of B cell specific transcription factors such as BOB-1, Oct-2 and PU.1 leading to the loss of B cell identity (Re et al., 2001, Torlakovic et al., 2001). This phenotype could be induced through EBV-dependent regulation of the cellular repressive complex Polycomb. Polycomb proteins silence many genes associated with growth and developmental regulation, including the p16<sup>INK4A</sup> and p14<sup>ARF</sup> promoters through H3K27Me3 methylation (for review see Chase and Cross, 2011). Polycomb-associated proteins KDM6B,

EZH2 and BMI-1 are all found to be upregulated in HL potentially by LMP1 (Dutton et al., 2007, Anderton et al., 2011, Dukers et al., 2004, Raaphorst et al., 2000). This may also contribute to the loss of B cell identity.

Reed-Sternberg cells over-express cytokines and chemokine receptors causing the influx and suppression of immune cells (Roullet and Bagg, 2007, Mani and Jaffe, 2009). Interestingly these signals maybe required for cell survival inside its microenvironment. For example Reed-Sternberg cells do not proliferate well in culture, nor can they persist in immunocompromised mice (Kuppers, 2009). A constitutively expressed chemokine in Reed-Sternberg cells, RANTES (CCL5), can recruit specific T cells which are required for cell survival and proliferation (Aldinucci et al., 2008). Reed-Sternberg cells also produce several cytokines such as galectin 1 and prostaglandin E<sub>2</sub>, which have been demonstrated to suppress T cell function and optimise microenvironment conditions (Juszczynski et al., 2007, Gandhi et al., 2007, Chemnitz et al., 2006).

#### **1.2.4.3. *Undifferentiated Nasopharyngeal Carcinoma (UNPC)***

Prevalent in China and South East Asia, undifferentiated Nasopharyngeal carcinoma (UNPC) is a cancer of nasopharyngeal epithelial cells and is strongly associated with EBV (Raab-Traub, 2002). Like HL, UNPC is characterised by the presence of a large lymphocyte infiltrate with a lower percentage of UNPC cells. EBV is present in a latency II state characterised by high expression of LMP1 LMP 2A, EBNA 1 and the EBERs (Brooks et al., 1992). Prior to EBV transformation, several precursors may be required for UNPC progression; such as a concentrated diet of Cantonese-style salted fish and several epigenetic modifications including chromosome deletions of the p16 gene locus (Yu et al., 1986, Lo and Huang, 2002). Associations between the immune response and transformed cells appear to be key to the progression of UNPC malignancy (Young and Rickinson, 2004). For example; both EBNA 1 and LMP1 have been shown to upregulate chemokine production (O'neil et al., 2008, Lai et al., 2010), recruiting T cells that augment the survival of the UNPC cell (Agathangelou et al., 1995, Sbihi-Lammali et al., 1999).



#### **1.2.4.4. *T cell Lymphoma***

EBV has also been detected in various T cell lymphomas (Brink et al., 2000). Both EBNA 1 and EBER 1 transcripts are detectable in 90% of nasal T cell lymphomas (Harabuchi et al., 2009). Since T cells do not express CD21 on their surface, the mechanism of EBV internalisation and infection is still unclear (Tsoukas and Lambris, 1993). However, infection may occur during T cell activation and eradication of EBV-infected cells (Brink et al., 2000). Interestingly, some EBV strains containing a 30bp deletion in the C terminus of the LMP 1 gene have been detected in T-cell lymphoma and possibly may play a role in immune evasion contributing to the development of the disease (Kim et al., 2003, Nagamine et al., 2007).

#### **1.2.4.5. *Infectious Mononucleosis (IM)***

Primary EBV infection usually occurs within early childhood between the ages of 1-5 and is asymptomatic. However if infection is delayed until adolescence, 50% of cases can result in IM (Henke et al., 1973). General symptoms include fatigue, fever, sore throat and malaise. EBV is usually spread through saliva and IM is associated with very high-salivary viral titres. IM is usually characterised by 0.1% to 1% EBV positive cells in the B cell pool, in rare cases it has been documented to be as high as 10% (Klein et al., 1976, Robinson et al., 1980). IM can be fatal if EBV-transformed B cells become dominant, as seen in immune-suppressed individuals (Falk et al., 1990).

B cells expressing all EBV latency types during IM can be detected (Fields et al., 2001). In response to delayed infection, a large hyper-activated T cell response is generated, targeting both lytic and latent proteins (Callan et al., 1996, Precopio et al., 2003). However, this over compensation generally contributes to the pathogenesis of the disease causing high T cell and antibody expression and T cell suppression (Tosato et al., 1985, Hadinoto et al., 2008). Clute *et al* demonstrated the activation of improper CD8+ T cells could contribute to disease pathogenesis (Clute et al., 2005). These T cells had specificity for influenza proteins and only partial specificity towards EBV proteins (Clute et al., 2005). The current treatment for IM is either Acyclovir, a nucleoside analogue that prevents viral replication or corticosteroids. Surprisingly,

the duration of EBV infection in IM patients using Acyclovir has been shown to be the same without it (Jenson, 2004). An EBV vaccine targeting the viral glycoprotein required to mediate cell entry, gp350, reduced the likelihood of IM-like symptoms during phase II clinical trials (Sokal et al., 2007).

#### **1.2.4.6. *Post-transplant lymphoproliferative disease (PTLD)***

Post-transplant lymphoproliferative disease (PTLD) occurs in 1-10% of transplant patients, with about 50% developing as a result of EBV infection (Haque et al., 1996, Ho et al., 1988a). Patients that require transplants or blood transfusions require immunosuppressive treatments in order to counter rejection. The resulting suppressed immune system can then lead to opportunistic diseases such as EBV-associated tumours. Therefore PTLD can occur in seronegative recipients receiving blood and/or organs from an EBV<sup>+</sup> donor (Alfieri et al., 1996, Cen et al., 1991, Gerber et al., 1969). The prognosis varies depending on which organ is being transplanted; heart, lungs or intestinal transplantations occur are associated with a 5-10% likelihood of developing PTLD (Webber et al., 2006, Dharnidharka et al., 2002). The disease progresses with similar symptoms to IM but fatality occurs in more than 50% of cases (Nalesnik, 1998). The age of the recipient is definitely a factor; children who acquire EBV lymphoma post-transplant have the highest mortality rates (Collins et al., 2001).

Initial treatment is the reduction in immunosuppressive therapy (Holmes and Sokol, 2002). An anti-CD20 mouse monoclonal antibody (rituximab) can also be used as an alternative therapy (Oertel et al., 2000). The CD20 cell surface receptor is expressed on B cells and is upregulated during PTLD. Interestingly, interactions with rituximab and the B cell receptor have been shown to cause apoptotic cellular signalling and may contribute to immunosuppression (Kheirallah et al., 2010, Franke et al., 2011). Immunosuppressive therapy, rituximab and chemotherapy are associated with a high success rate and PTLD clearing, however no therapy is associated with 100% remission (Dharnidharka and Araya, 2009).

### **1.2.5. The EBV latent genes**

#### **1.2.5.1. EBNA 2**

EBNA 2 is a transcriptional regulator of both viral and cellular genes. The protein consists of 9 conserved regions (CR1-9), and separate polyproline and a poly arginine-glycine (Poly-RG) repeat regions (Fields et al., 2001). The role of EBNA 2 during infection was determined from two key studies using the P3HR-1 virus. P3HR-1 is a naturally occurring strain of EBV that contains both an EBNA 2 and part EBNA-LP gene deletion. The essential role of EBNA 2 in B cell immortalisation was elucidated when recombinant P3HR-1 viruses failed to transform B cells (Cohen et al., 1989). Experiments using the P3HR-1 strain were also key in demonstrating that continual growth of LCLs was EBNA 2 dependent. The EBNA 2 gene was fused to an estrogen receptor, cloned into a mini-EBV vector and used to complement P3HR1 virus, creating a conditionally active EBNA 2 cell line (ER/EB) (Kempkes et al., 1995b). These ER/EB cells only maintained their growth in the presence of active estrogen/EBNA 2 (Kempkes et al., 1995b).

EBNA 2 cannot directly bind to DNA, instead EBNA 2 utilizes the cellular Notch-pathway adapter protein CBF1 (RBP-Jk) to bind upstream of and activate the latent promoters C (Sung et al., 1991, Jin and Speck, 1992), LMP1, LMP2A and LMP2B (Fahraeus et al., 1990a, Wang et al., 1990b, Ghosh and Kieff, 1990). Subsequently EBNA 2 has been shown to interact *in vivo* with each promoter using Chromatin immunoprecipitation assays (ChIP) (Bark-Jones et al., 2006, Alazard et al., 2003). The RBP-Jk protein generally binds to the DNA sequence GTGGGAA (Tun et al., 1994) to repress transcription through the recruitment of co-repressor complexes to condense DNA and prevent transcription factor access. These co-repressor complexes have been shown to contain the SKIP protein, the histone deacetylases HDAC1 and HDAC2, Sin3A and a silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT) (Kao et al., 1998, Zhou and Hayward, 2001, Zhou et al., 2000a). Further experiments suggested that without EBNA 2, SMRT contacts both RBP-Jk and SKIP to recruit the repressive complex (Zhou and Hayward, 2001, Zhou et al., 2000a). Like Notch proteins, EBNA 2 activates transcription by binding to both RBPJk and SKIP simultaneously to displace repressive

complexes to activate transcription (Zhou et al., 2000b, Zhou et al., 2000a). Two isoleucine residues (307 and 308) contained within the EBNA 2 conserved region 5 (CR5) are critical for SKIP interactions (Zhou et al., 2000a, Yalamanchili et al., 1994). The CR6 region of EBNA 2 (residues 318 to 327) is crucial for the interaction between EBNA 2 and RBP-Jk (Ling and Hayward, 1995, Ling et al., 1993). Mutational analysis of two key tryptophan residues (amino acids 323 and 324) abolished both RBP-Jk binding and subsequent Cp activation (Ling et al., 1993). In addition, mutant RBPJk studies reduced LMP1p transactivation by 60% and demonstrated that the B cell specific transcription factor PU.1 was required for EBNA 2-dependent LMP1p transcription (Johannsen et al., 1995, Sjoblom et al., 1995).

In addition to viral genes, microarrays carried out using EBNA 2 conditional LCLs or EBV<sup>+</sup> BL cells expressing only EBNA 2 have shown EBNA 2 to regulate hundreds of cellular genes involved in numerous cellular processes (Thompson et al., 1999, Cahir-Mcfarland et al., 2004, Maier et al., 2006). Most of these targets are upregulated by EBNA 2 by unknown mechanisms. Initial studies identified the upregulation of CD21 by EBNA 2 (Cordier et al., 1990) but the mechanism of gene targeting is still unknown. Other cellular EBNA 2 targets include the B cell activation marker CD23 (Wang et al., 1987a), the proto-oncogene c-MYC (Kaiser et al., 1999), the B cell transcription factor RUNX3 (Spender et al., 2002) and the G1 cyclin, Cyclin D2 (Sinclair et al., 1994). 36 hrs post-infection CD21, CD23 and c-MYC mRNAs are all induced by EBNA 2 (Alfieri et al., 1991). Interestingly, microarrays also identified many genes that were downregulated by EBNA 2, including the B-cell receptor genes CD79A and CD79B and the transcriptional regulator of germinal centre B cells BCL6 (Thompson et al., 1999, Cahir-Mcfarland et al., 2004, Maier et al., 2006). Furthermore, in BL cell lines, EBNA 2 has been shown to down-regulate the translocated proto-oncogene c-MYC (Jochner et al., 1996). Currently, there is no proposed mechanism for EBNA 2-dependent repression of transcription.

Two repeated regions (poly-proline and poly-RG) are present within the EBNA 2 protein with unknown functions. Firstly, in a region spanning 45 amino acids close to the N terminus, 43 proline residues define the polyproline region (Dambaugh et al., 1984). This region is expendable for transformation activity, but may have a role in LMP1p regulation (Gordadze et al., 2002). Similarly, the poly-arginine glycine repeat region (poly-RG), spanning amino acids

337 to 354 is critical but not essential for EBNA 2-dependent immortalisation (Tong et al., 1994). In fact reporter assays in which the poly-RG region was deleted resulted in enhanced LMP1p activation by EBNA 2 by up to 4-fold (Tong et al., 1994). Consistent with a potential regulatory role, EBNA 2 is methylated *in vivo* in its poly-RG region which can interact with the survivor motor neuron protein (SMN) to upregulate LMP1 expression (Barth et al., 2003, Voss et al., 2001). SMN facilitates the assembly of spliceosome components, therefore it could bind EBNA 2 to stimulate LMP1 transcription through enhanced splicing activity (Barth et al., 2003). In addition, *in vitro* associations with histone H1 have been detected although functional consequences *in vivo* are to be determined (Tong et al., 1994).

EBNA 2 residues 3-30 are critical for LMP1p transcription and for maintaining growth (Gordadze et al., 2004). This region is required for co-activation with EBNA-LP, although EBNA 2 and EBNA-LP may not co-immunoprecipitate together (Gordadze et al., 2004, Peng et al., 2005). The latency III transcriptional programme is associated with expression of all the EBNAs which augment proliferation and enable cells to be more apoptosis resistant (Gregory et al., 1991). In response to pro-apoptotic signals, the transcription factor Nur77 migrates to the mitochondria to stimulate cytochrome c release (Li et al., 2000). EBNA 2 has been shown to interact directly with Nur77 through its CR4 region (amino acids 123-147) and inhibit cytochrome c transcription (Lee et al., 2002). Similarly, the Notch1C downregulates the Nur77 apoptotic pathway through direct interactions in T cells (Jehn et al., 1999).

#### **1.2.5.1.1. Transcriptional regulation by EBNA 2**

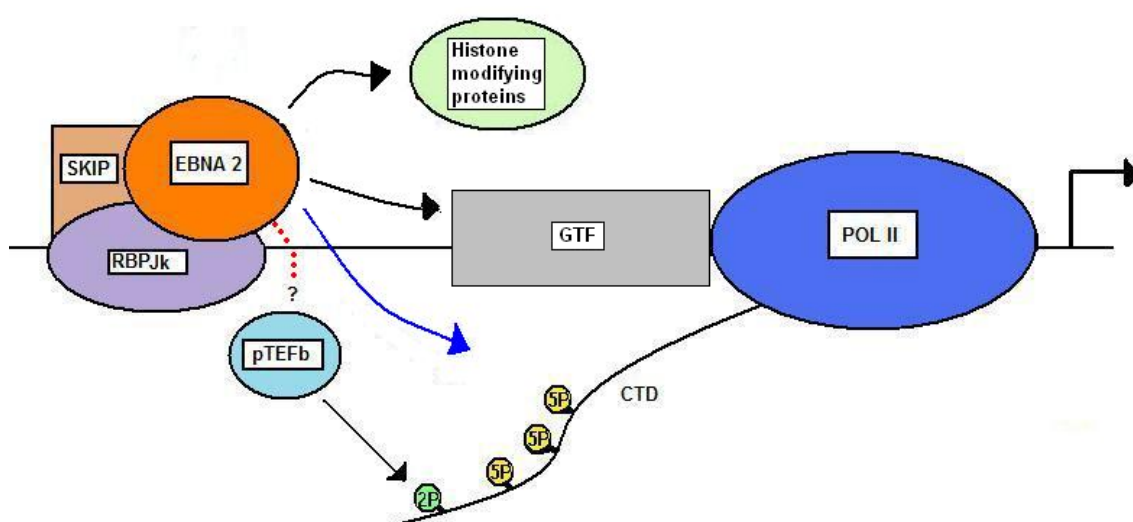
Once recruited to promoters, activators can recruit co-activator complexes through transactivator domains to stimulate transcription by modifying histone tails and/or augmenting Pol II recruitment. EBNA 2 contains a trans-activating domain (TAD) in its C-terminus (amino acids 449 to 462), which is essential for transformation function (Cohen and Kieff, 1991, Cohen et al., 1991). The Papillomavirus E7 protein, known to bind the retinoblastoma protein (Rb), is partly homologous (29 amino acids) to the EBNA 2-TAD (Dyson et al., 1989). However, unlike E7, EBNA 2 has not been shown to interact with Rb.

The EBNA 2-TAD does not directly recruit Pol II or TBP to promoters, instead it interacts with the GTFs TFIIH (p62 and XPD) (Tong et al., 1995a), TFIIIE (p100) (Tong et al., 1995b), TAF40 and TFIIB (Tong et al., 1995c). In addition the EBNA 2-TAD can recruit histone modifying complexes to stimulate transcription. Interactions with HATs such as p300, CBP and PCAF have all been documented (Wang et al., 2000). This is supported by ChIP assays that showed increased histone acetylation at Cp and LMP1p in conjunction with EBNA 2 binding (Alazard et al., 2003). Mutational studies have shown that if tryptophan 454 is mutated to an alanine or threonine residue, EBNA 2 is unable to interact with any of these proteins (Tong et al., 1995b, Tong et al., 1995a, Tong et al., 1995c, Wang et al., 2000). EBNA 2 also binds to the chromatin remodelling complex hSNF5 independently of its TAD (Kwiatkowski et al., 2004). This interaction required a TATA box, indicating that several other factors may also be needed to stabilise the interaction (Wu et al., 1996).

Work from our laboratory has demonstrated Pol II serine 5 CTD phosphorylation at Cp was increased on activation in the conditional ER/EB cell line (Bark-Jones et al., 2006). Furthermore, both Cp and LMP1p transcription was dependent on the elongation factor and CTD kinase pTEFb in transient reporter assays (Bark-Jones et al., 2006). Therefore EBNA 2-dependent activation of viral and cellular transcription is mediated through the indirect recruitment of the transcription machinery through interactions with basal transcription components, stimulation of histone acetylation and remodelling and Pol II serine 5 CTD phosphorylation (Fejer et al., 2008, Day et al., 2007, Bark-Jones et al., 2006) (see Figure 2).

EBNA 2 transcriptional activity is regulated by phosphorylation (Grasser et al., 1992). Yue *et al* demonstrated CDK1-dependent hyperphosphorylation of EBNA 2 coincided with a decreased affinity with the adaptor protein PU.1 and a decrease in LMP1 transcripts during M phase (Yue et al., 2004). Follow-up experiments revealed CDK1 could phosphorylate EBNA 2 at serine 243 *in vitro* and *in vivo* causing a decrease in Cp activation (Yue et al., 2006). During lytic cycle, latency proteins are downregulated to augment the induction of lytic genes and replication. Yue *et al* investigated a potential interaction and regulation of EBNA 2 and the EBV serine/threonine lytic protein kinase (PK) BGLF4 (Yue et al., 2005). Similarly to CDK1, BGLF4 could bind and phosphorylate EBNA 2 on serine 243 resulting in decreased transcriptional function (Yue et al.,

2005). Serine 243 is located close to the CR5 and CR6 regions of EBNA 2, which is required for the recruitment of EBNA 2 to promoters (Ling and Hayward, 1995). Therefore the addition of negatively charged phosphate groups could induce a conformational change in CR5, preventing key residues from contacting the adapter proteins required for transcription. Other residues targeted for phosphorylation and crucial for EBNA 2 function are serines 469 and 470, located in the TAD. Phosphorylation by a cell cycle enzyme casein kinase II resulted in an association with the chromatin remodelling complex hSNF5 (Kwiatkowski et al., 2004). This subsequent phosphorylation was required to maintain growth transformation (Kwiatkowski et al., 2004). ‘



**Figure 2 Mechanisms of EBNA 2 activation of transcription.** EBNA 2 is recruited upstream of promoters through interactions with cellular adapter proteins. Interactions with histone modifiers and the general transcription factors (GTF) are indicated with black arrows. The blue arrow indicates an indirect mechanism where EBNA 2 binding to promoters may facilitate serine 5 phosphorylation on the CTD.

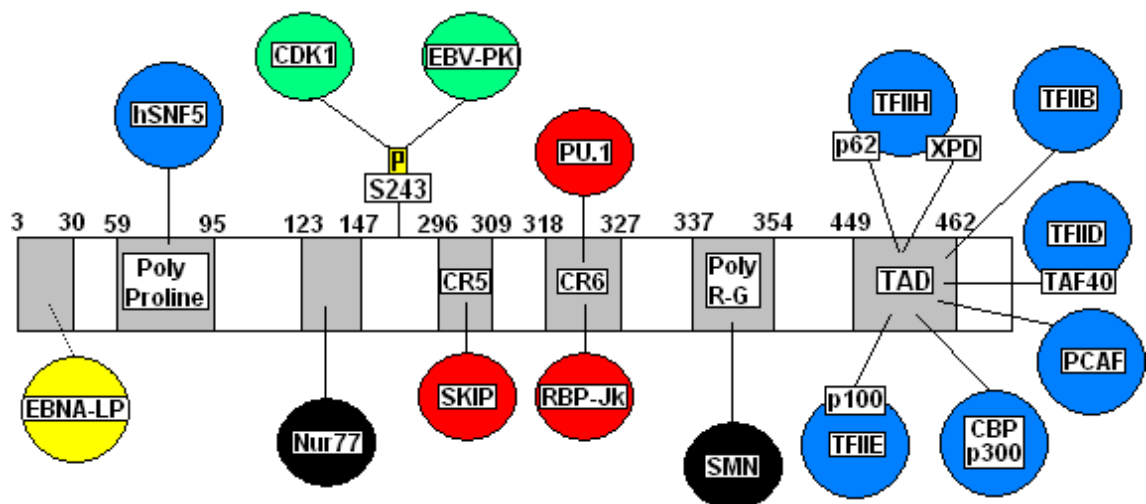
### **1.2.5.1.2. Type 1 and Type 2 EBNA 2**

EBV strains have been characterised as either type 1/A or type 2/B. The type 1 EBV strains are distributed globally, whereas type 2 EBV is generally localised to smaller geographical areas such as Central Africa and New Guinea (Zimber et al., 1986, Abdel-Hamid et al., 1992). Both EBV types can transform B cells *in vitro*, however type 1 strains can establish transformants much more efficiently than type 2 (Rickinson et al., 1987). Type 1 EBV is also more likely to cause IM than type 2 (Crawford et al., 2006). A major difference between viral types is the EBNA 2 gene sequence, which shares only 47% homology between the two strains (Addinger et al., 1985, Dambaugh et al., 1984). There is some sequence variation between the EBNA 3 family and EBNA-LP, although to a lesser extent (Sample et al., 1990, Dambaugh et al., 1984).

Recombinant type 1 viruses containing a type 2 EBNA 2 sequence transformed B cells slowly and similar to a wild-type, type 2 virus (Cohen et al., 1989). Similarly recombinant type 2 viruses containing a type 1 EBNA 2 sequence could transform B cells comparably to a type 1 wild-type virus (Cohen et al., 1989). Therefore EBNA 2 is the key factor for growth transformation between the two EBV strains. Most EBNA 2 sequence variation is located within the N-terminal half of the protein, which contains two divergent regions and the polyproline domain and contributes to the size difference between the two proteins (Cohen et al., 1991).

To determine the transcriptional differences between type 1 and type 2 EBNA 2, conditional fusion proteins of type 1 or type 2 EBNA 2 were transiently transfected into BL cells (Lucchesi et al., 2008). Interestingly, type 1 EBNA 2 induced LMP1 quicker and in larger quantities when compared to type 2 EBNA 2 (Lucchesi et al., 2008). Furthermore, type 1 EBNA 2 could also induce several cellular genes such as the chemokine receptor *CXCR7* and the integrin receptor *ADAMDEC1* more efficiently (Lucchesi et al., 2008). Recombinant growth assays determined the key type 1 EBNA 2 sequences required to compensate for type 2 deficiency in B cell proliferation (Cancian et al., 2011). These experiments revealed the C-terminal region of type 1 EBNA 2 that encompassed the poly-RG, conserved region 7 (amino acids 376-425) and the EBNA 2-TAD were all required in tandem to mimic the type 1 proliferation phenotype





**Figure 3 The EBNA 2 protein.** A schematic of the EBNA 2 protein indicating its known domains and protein interactions therein. The numbers shown indicate the amino acid numbers and define areas of interaction. Known interactions are defined by black lines linking the protein to the particular area. Possible interactions are shown by broken lines. S243 indicates the serine 243 residue that is critical for EBNA 2-DNA binding. CR = conserved region, Poly-RG = arginine-glycine repeat region and TAD = Transcriptional activation domain

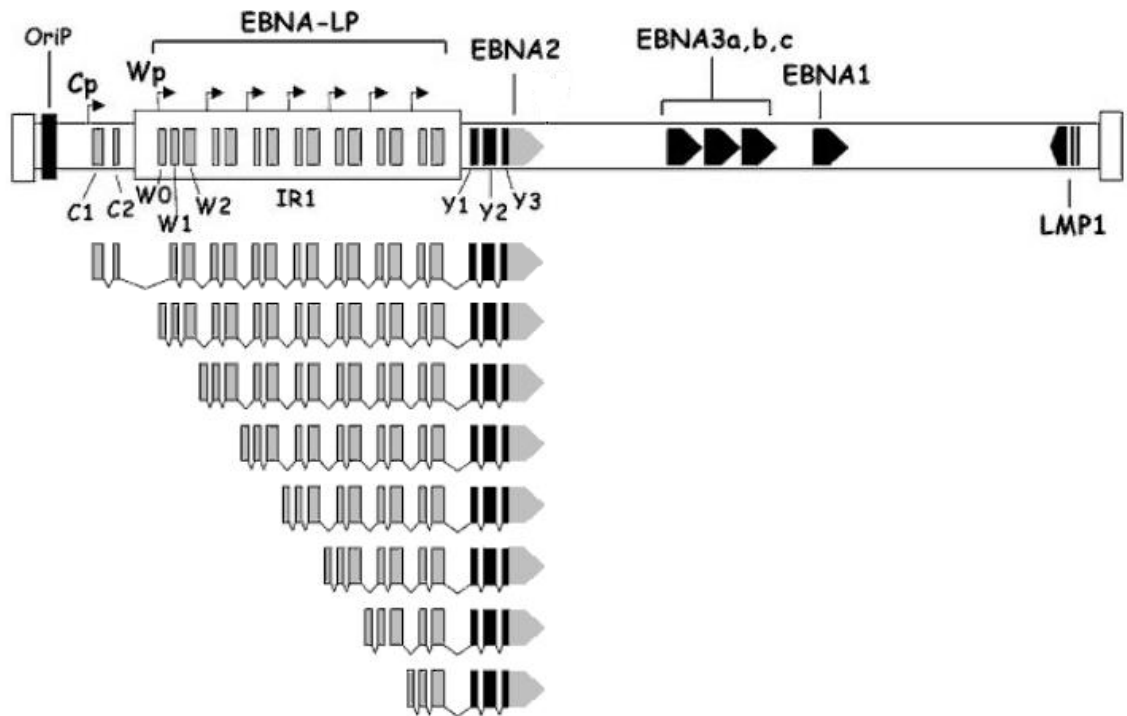
(Cancian et al., 2011). Interestingly all 3 regions are associated with the activation of LMP1 transcription in conjunction with the EBNA 2 co-activator EBNA-LP (Cancian et al., 2011).

#### **1.2.5.2. EBNA-LP**

EBNA-LP is usually detectable between 10-12 hours post EBV infection (Allday et al., 1989, Alfieri et al., 1991) and is required for efficient B cell immortalisation (Allan et al., 1992, Mannick et al., 1991). During early infection different isoforms of varying lengths are detected because the entire EBNA-LP coding sequence is contained within the W repeat region (IR1) (Speck et al., 1986, Wang et al., 1987b). Each W repeat extends the EBNA-LP protein by an extra 66 amino acids (Sample et al., 1986, Finke et al., 1987). The size of EBNA-LP transcripts depends on the number of W repeats contained within the particular episome, the initial Wp from where transcription has been initiated and the variable splicing of exons W<sub>1</sub>/W<sub>2</sub>. The significance of EBNA-LP length variation is unknown. A minimum of two W<sub>1</sub>W<sub>2</sub> repeats are required for immortalisation and EBNA-LP containing only one W<sub>1</sub>W<sub>2</sub> repeat is restricted exclusively to the cytoplasm (Yoo et al., 1997, Tierney et al., 2007, Peng et al., 2000b)

The main role of EBNA-LP during infection is to co-activate EBNA 2-dependent genes. CAT (chloramphenicol acetyltransferase) reporter assays containing upstream Cp or LMP1p were enhanced by EBNA 2 and EBNA-LP co-expression by 10-30 fold compared to EBNA 2 expression alone (Harada and Kieff, 1997). Interestingly in the absence of EBNA 2, EBNA-LP repressed transcription from LMP1p and Cp (Harada and Kieff, 1997). Transient transfection assays also confirmed EBNA-LP could co-activate the LMP1p between 5-10 fold when co-expressed with EBNA 2 (Nitsche et al., 1997). This function of EBNA-LP is not limited to just viral genes, it has also been shown to co-stimulate EBNA 2-dependent transcription of the Cyclin D2 gene (Sinclair et al., 1994) and the transcription factor HES1 (Portal et al., 2011).

The regulation of EBNA-LP co-activation function by phosphorylation has been well documented. Initially, Kitay and Rowe demonstrated that EBNA-LP could be phosphorylated *in*



**Figure 4 The relationship between Wp splicing and EBNA-LP gene transcripts.** Schematic illustrating the multiple transcripts that can initiate from Wp or Cp to produce EBNA-LP transcripts of varying lengths. Each EBNA-LP transcript contains at least one IR1 repeat ( $W_1$  and  $W_2$  exon) and the  $Y_1Y_2Y_3$  exons encompassing the EBNA 2 gene. Splicing may also skip neighbouring IR1 repeats to truncate EBNA-LP further (not shown). The location of OriP, other EBNA genes and LMP1p are also indicated. Adapted from (Speck, 2005)

*vitro* by CDK1 and casein kinase II (Kitay and Rowe, 1996). Mutagenesis studies then identified serine 35 (S35) as a key target residue; its phosphorylation led to enhanced LMP1p transcription (Yokoyama et al., 2001, Peng et al., 2000b, Mccann et al., 2001). A site that is found in every W<sub>2</sub> exon and is highly conserved amongst other lymphocryptoviruses (Peng et al., 2000a, Mccann et al., 2001). Kato *et al* confirmed *in vivo* phosphorylation of EBNA-LP S35 was regulated by CDK1 and the EBV protein kinase BGLF4 (Kato et al., 2003). Other mutants unable to co-activate the LMP1p were defective in nuclear localisation (Peng et al., 2000b).

The precise mechanism of EBNA-LP co-activation is not fully defined and may not involve direct interactions with EBNA 2 (Peng et al., 2005). EBNA-LP may bind and sequester the repressive factors recruited by RBP-Jk away from promoters (Portal et al., 2011, Portal et al., 2006). EBNA-LP was shown to bind to and remove co-repressor complexes such as HDAC4 and NCoR from EBNA-dependent promoters, resulting in the enhanced association between EBNA 2 and RBPJk at promoters (Portal et al., 2011, Portal et al., 2006).

### **1.2.5.3. LMP1**

LMP1 is a potent growth inducer of epithelial cells, rat fibroblasts and B-cells and is critical for *in vitro* B-cell immortalisation during EBV infection (Fahraeus et al., 1990b, Kaye et al., 1993, Wang et al., 1985). LMP1 is a functional, constitutively active CD40 homologue that stimulates both cellular growth and survival pathways (Middeldorp and Pegtel, 2008, Homig-Holzel et al., 2008). It is a transmembrane protein, consisting of 6 membrane-spanning regions and two highly acidic C-terminal activation regions (CTAR-1 and CTAR-2) that act as docking sites for signalling molecules (Fennewald et al., 1984, Huen et al., 1995). Both CTARs mediate the activation of signalling pathways. Only 30% of LMP1 resides in the plasma membrane, where it forms aggregates crucial for functionality (Hennessy et al., 1984, Liebowitz et al., 1986, Robertson, 2010).

CTAR-1 associates with tumour necrosis factor receptor associated factors (TRAFs), interactions that are critical for B-cell immortalisation (Izumi et al., 1997). Mutation of two tyrosine residues within CTAR-2 also can inhibit LCL outgrowth (Izumi and Kieff, 1997). Further experiments identified these tyrosine residues were required for tumor necrosis factor receptor-

associated death domain protein binding (TRADD) (Izumi and Kieff, 1997). Through the TRADD and TRAF proteins, the CTARs upregulate the NF- $\kappa$ B pathways (Atkinson et al., 2003, Paine et al., 1995, Saito et al., 2003, Izumi et al., 1999, Izumi and Kieff, 1997). By overexpressing a cellular inhibitor of the NF- $\kappa$ B pathways, NF- $\kappa$ B activation was shown to be essential for growth and survival of LCLs (Cahir-Mcfarland et al., 2000). This could explain why both CTAR regions of LMP1 differentially regulate NF- $\kappa$ B signalling.

Apart from the NF- $\kappa$ B pathways, LMP1 activates other signaling pathways involved in growth and survival. In particular, LMP1-dependent upregulation of pro-survival proteins BCL-2 and A20 prevent activation of the p53-mediated apoptosis pathway (Fries et al., 1996, Okan et al., 1995). The p38 MAPK signaling pathway can be upregulated through either CTAR-1 or CTAR-2 (Eliopoulos et al., 1999). Additionally, amino acids 378 to 386 of CTAR-2 are required to activate JNK signaling pathway (Dawson et al., 2003). Co-expression of both EBNA 2 and LMP1 induces the B cell activation marker CD23 (Wang et al., 1990a). Presumably LMP1 acts through signalling pathways since EBNA 2 and LMP1 are located in different cellular compartments. Activated in lytic cycle, a third LMP1 promoter encodes a truncated form of LMP 1 (D1LMP1) which is unable to immortalise fibroblasts (Wang et al., 1988). The exact function of D1LMP1 is unknown but it may contribute to cell survival.

#### **1.2.5.4. LMP 2A**

LMP2A is a membrane protein expressed during latency but not essential for growth transformation of B cells (Longnecker et al., 1992). It contains 12 membrane-spanning domains and a 27 amino acid cytoplasmic tail important for signalling (Longnecker et al., 1991). Immunofluorescence experiments demonstrated LMP2A co-localises with LMP1 in patches on the plasma membrane, possibly to increase LMP1 signalling (Longnecker and Kieff, 1990). The B cell receptor (BCR) regulates proliferation and immune responses through the recruitment and activation of the src family of kinases and calcium mobilisation (for review see Gauld and Cambier, 2004). LMP2A inhibits BCR signalling by either competing for the src kinases, or by recruiting E3 ubiquitin ligases to the plasma membrane leading to proteasome-dependent degradation (Winberg et al., 2000, Busson et al., 1995, Fruehling and Longnecker, 1997).

Microarrays have shown both EBNA 2 and LMP1 downregulate BCR components CD79A and CD79B (Cahir-Mcfarland et al., 2004, Maier et al., 2006). Since a functional BCR is required for B cell survival, LMP2A has also been shown to mimic BCR signalling through the association with MAPK/ERK1 or through calcium mobilisation (Young and Rickinson, 2004, Panousis and Rowe, 1997, Alber et al., 1993, Beauvils et al., 1993). This supplementation of BCR signalling maybe required during GC reactions since cells with a faulty BCR undergo apoptosis (Kurosaki, 2002). Transgenic mouse experiments have shown LMP2A expression, in the absence of the BCR, enhances B cell survival during the GC reaction (Caldwell et al., 1998, Caldwell et al., 2000).

LMP2A has also been implicated in the maintenance of latency by blocking BCR-dependent lytic cycle reactivation. LMP2A inhibits BCR signalling by preventing increases in intracellular calcium and the activation of BCR-dependent genes, which are known activators of the EBV lytic cycle inducer Zta (Miller et al., 1993, Miller et al., 1995). Further experiments demonstrated transformed LCLs carrying a null LMP2A could not prevent lytic cycle induction through calcium flux (Miller et al., 1994).

Interestingly, when expressed in epithelial cells, LMP2A possesses growth transforming abilities, possibly through the constitutive activation of both the PI3 kinase and Ras pathways (Scholle et al., 2000, Fukuda and Longnecker, 2007). Both of these pathways are not activated by LMP2A in B cells, suggesting cell specific factors are involved. Furthermore, high levels of LMP2A are expressed in UNPC (Lennette et al., 1995, Busson et al., 1992). LMP2A expression in UNPC stimulates induction of the integrin *ITGa6* causing cells to migrate (Pegtal et al., 2005). Therefore LMP2A could promote metastasis in UNPC patients, possibly explaining why higher LMP2A expression in UNPC correlates to a lower patient survival rate (Pegtal et al., 2005).

#### **1.2.5.5. EBNA 1**

EBNA 1 is the only latent protein to be expressed in all EBV-associated diseases, including BL. EBNA 1 is a multifunctional viral protein, required for the immortalisation of B cells (Humme et al., 2003). Through interactions with OriP binding elements, EBNA 1 regulates viral

replication, chromosome segregation and transcription (Yates et al., 1985, Lupton and Levine, 1985, Sugden and Warren, 1989, Yates et al., 1984, Rawlins et al., 1985).

The origin of latent replication (OriP) is situated between the EBV terminal repeats and Cp. It contains two EBNA 1 binding elements, DS (dyad symmetry) and FR (family of repeats) (Reisman et al., 1985). EBNA 1 can bind both elements through its C-terminal domain, amino acids 459-607 (Ambinder et al., 1991, Chen et al., 1993). The DS element contains four EBNA 1 binding sites, 2 of which are critical for viral replication (Wysokenski and Yates, 1989, Koons et al., 2001). Once bound to DS, EBNA 1 interacts with two cellular complexes that facilitate viral replication, the cellular origin recognition complex (ORC) and the minichromosome maintenance complex (MCM) (Chaudhuri et al., 2001, Schepers et al., 2001). Mutations in either the ORC and MCM complexes or the DS sequence fails to initiate replication (Chaudhuri et al., 2001, Dhar et al., 2001, Schepers et al., 2001).

The FR of OriP is required for mitotic segregation and transcriptional function (Reisman and Sugden, 1986). Mitotic segregation occurs once every cell cycle during mitosis and only requires EBNA 1 and FR (Lee et al., 1999). During mitosis, EBNA 1 tethers the partitioned EBV episome to highly condensed host cell chromosomes (Petti et al., 1990, Kanda et al., 2001). This process ensures the EBV episome is copied and retained resulting in lifelong persistence. Detailed mutational studies revealed amino acids 325 to 376 of EBNA 1 were required for tethering (Shire et al., 2006, Hung et al., 2001). This region of EBNA 1 coincides with four repeat regions, each 8 amino acids in length, consisting of glycine-arginine repeats and a serine residue (GGGGRGRGGS) (Polvino-Bodnar et al., 1988). Serine phosphorylation and arginine methylation were required for episomal segregation but not for replication (Shire et al., 2006, Shire et al., 1999).

The binding of EBNA 1 to FR can function to enhance transcription from both Cp and LMP1p (Sugden and Warren, 1989, Reisman and Sugden, 1986, Gahn and Sugden, 1995). Moreover, the binding of EBNA 1 homodimers to FR appears to be required for the full activation of Cp (Zetterberg et al., 2004). When Cp and Wp are inactive (latency I and II), EBNA 1 is expressed from and regulates a downstream promoter called Qp (Schaefer et al., 1995, Tsai et al., 1995). In BL cells, EBNA 1 was shown to bind and activate Qp through two

downstream sequences (Nonkwelo et al., 1996, Schaefer et al., 1995). Similarly, the E2F transcription factor E2F-1 has also been shown to upregulate Qp-dependent EBNA 1 transcription through the same binding sites (Sung et al., 1994). Recently, chromosomal conformational capture (3C) techniques demonstrated OriP to be in close proximity to both active C and Q promoters during different EBV latencies, suggesting DNA looping may play an integral part in EBNA 1-dependent enhancer function (Tempera et al., 2011). Additionally *Lin et al* showed an interaction between EBNA 1 and the chromatin adapter protein Brd4 at OriP, which stimulated transcription (Lin et al., 2008).

#### **1.2.5.6. The EBNA 3 family of proteins**

The EBNA 3 family of genes (3A, 3B and 3C) are located in tandem in the EBV genome and the proteins share approximately 30% homology (Shimizu et al., 1988, Ricksten et al., 1988, Jiang et al., 2000). The EBNA 3 proteins function as transcriptional regulators and deregulators of the cell cycle. Each EBNA 3 protein can bind independently to the RBPJk adapter protein and prevent EBNA 2-transcriptional activation in reporter assays (Robertson et al., 1995, Robertson et al., 1996, Waltzer et al., 1996, Le Roux et al., 1994, Zhao et al., 1996). In the context of latent infection, EBNA 3C has not been shown to have any significant regulatory input on Cp (Jimenez-Ramirez et al., 2006). However, EBNA 3C has been shown to increase LMP1p transcripts both *in vitro* and *in vivo* possibly through interactions with PU.1 (Jimenez-Ramirez et al., 2006, Marshall and Sample, 1995).

Primary B cells infected with EBV containing premature stop codons in either EBNA 3A or 3C showed that both proteins were required for *in vitro* immortalisation (Tomkinson et al., 1993). Conversely, similar studies showed EBNA 3B was dispensable for both immortalisation and growth (Tomkinson and Kieff, 1992). Recent EBNA 3A and 3C conditional-expression experiments investigated the relationship between growth transformation and RBP-Jk binding. Maruo *et al* demonstrated that the EBNA 3A protein required amino acids 170-240 to bind to RBP-Jk and maintain LCL growth (Maruo et al., 2005). Likewise, EBNA 3C required amino acids 180-231 to interact with RBP-JK and maintain LCL growth (Maruo et al., 2009). These data indicated that both EBNA 3A and 3C regulated LCL proliferation through gene transcription



dependent on interactions with RBP-Jk (Maruo et al., 2005, Maruo et al., 2009). Further experiments demonstrated that other amino acid mutations in both EBNA 3A and 3C retained binding to RBP-Jk sites but were unable to repress EBNA 2-dependent activation or sustain LCL growth, therefore both EBNA 3A and 3C regulate LCL growth in both a RBP-Jk dependent and independent way (Maruo et al., 2005, Maruo et al., 2009). By binding RBP-Jk, EBNA 3C and possibly EBNA 3A and 3B may recruit HDAC proteins (HDAC 1 and 2) in conjunction with large repressive complexes associated with deacetylase activity to silence EBNA 2-dependent transcription (Knight et al., 2003).

Recently, microarrays have identified a large number of cellular genes involved in apoptosis, cell cycle, cell migration and B-cell transcription factors that are differentially regulated in EBNA 3A (210), EBNA 3B (598) or EBNA 3C (839) knockout BL cell lines (White et al., 2010). Interestingly, 390 of the 1201 genes that were differentially regulated, required multiple EBNA 3 proteins to co-regulate expression (White et al., 2010). Three of these observed genes (p16<sup>INK4A</sup>, p14<sup>ARF</sup> and Bim) are tumour suppressors studied in more detail. The p16<sup>INK4A</sup> protein regulates the phosphorylation of the Retinoblastoma protein (pRb) and therefore the G<sub>1</sub>S checkpoint through binding and inactivation of CDK4 and CDK6 (Lukas et al., 1995, Koh et al., 1995, Medema et al., 1995). Once expressed, p14<sup>ARF</sup> prevents the polyubiquitination and degradation of p53 by binding and sequestering the p53 inhibitor protein MDM2 (Pomerantz et al., 1998). Both genes are regulated by Polycomb protein complexes, which silence transcription through the repressive H3K27Me3 methylase EZH2 (for review see Chase and Cross, 2011).

Using conditionally-expressing LCLs, EBNA 3C inactivation was shown to decrease pRb hyperphosphorylation and the accumulation of P16<sup>INK4a</sup> preventing cell cycle progression (Maruo et al., 2006). Further experiments identified a correlation between both EBNA 3A and 3C expression with the increased repressive epigenetic marker H3K27Me3 and consequent p16<sup>INK4A</sup>/ p14<sup>ARF</sup> repression (Skalska et al., 2010, Maruo et al., 2011). Repression was dependent on the C-terminal binding protein (CtBP), a known negative transcriptional regulator of the p14<sup>INK4A</sup> gene locus (Skalska et al., 2010). The third example of co-regulation is the pro-apoptotic, Bcl-2 protein Bim which regulates apoptosis through the activation of Bax

(Gavathiotis et al., 2008). Upon EBV infection Bim expression is repressed by H3K27Me3 epigenetic silencing (Paschos et al., 2009). EBNA 3A and 3C were shown to co-operatively downregulate Bim expression (Anderton et al., 2008). These data collectively suggest that the EBNA 3 family of proteins may co-regulate genes and cell survival through the utilisation of polycomb proteins and histone modifications (White et al., 2010).

The EBV C promoter encodes a long transcript of ~120 kb in length that is differentially spliced into all the EBNA proteins critical for the immortalisation of B cells. Therefore the transcription factors and other mechanisms utilised to regulate Cp are of utmost importance in EBV pathogenesis. EBNA 2 is the main latent viral transcription factor, it is responsible for the regulation of all latency III protein-encoding viral promoters (including Cp). In this study we investigated the presence of specialised transcription factors such as DSIF, NELF and pTEFb at all EBNA 2-dependent promoters and how the differential methylation of EBNA 2 affects its transcription functionality.

## 2. Materials and Methods

### 2.1. Tissue culture

All reagents were purchased from Fisher Scientific unless stated.

#### ***2.1.1. Tissue culture media and supplements***

##### **5,6-Dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB)** (Sigma)

Stored as a 100 mM stock in DMSO at -20°C.

##### **100x Penicillin-Streptomycin-Glutamine (PSG)** (Invitrogen)

Contains 100 units/ml penicillin G, 100  $\mu$ g/ml streptomycin sulphate and 29.2 mg/ml L-glutamine with 0.85% saline and 10 mM citrate buffer. Stored in 5 ml aliquots -20°C.

##### **Accell delivery media siRNA with 2mM L-glutamine** (Dharmacon)

##### **Dimethyl Sulphoxide (DMSO)** (Sigma)

##### **Fetal Bovine serum (FBS)** (Gibco)

Pre-screened for endotoxins ( $\leq 5$  EU/ml), haemoglobin ( $\leq 10$  mg/dl) levels and heat inactivated at 56°C for 30 mins. Stored in 50ml aliquots at -20°C.

##### **Flavopiridol (Flavopiridol hydrochloride)** (Sigma)

Stored as a 100  $\mu$ M stock in DMSO at 4°C.

##### **Freezing mix**

80% RPMI media (supplemented with 10% FBS and PSG), 10% FBS and 10% DMSO

##### **JQ1** (Gift from Stefan Knapp)

Stored as a 1  $\mu$ M stock at -20°C.

**Dulbecco's Phosphate Buffered saline without  $\text{CaCl}_2$  and  $\text{MgCl}_2$  (PBS)** (Invitrogen)

**RPMI 1460 media without L-glutamine** (Invitrogen)

## **2.1.2. Maintenance of cell lines**

### **2.1.2.1. Burkitt's Lymphoma cell lines (BL)**

Mutu I (clone 179) is an EBV type I cell line derived from a 7-year old African black male with Burkitt's Lymphoma that displays a latency I gene expression pattern (Gregory et al., 1990). Mutu III (clone 48) cells were derived from Mutu I cells that drifted in culture to display a group III latency gene expression profile (Gregory et al., 1990). Both cell lines were kindly provided by Martin Rowe and were passaged 1:5 twice weekly in RPMI media supplemented with 10% FBS and 5% PSG.

Akata cells are an EBV type I infected latency I Burkitt's Lymphoma cell line derived from a Japanese patient (Takada et al., 1991). They were passaged identically to Mutu cells and were provided by Alison Sinclair.

### **2.1.2.2. LCLs**

Lymphoblastoid cell lines PER 253 B95-8 LCL, PER 142 B95-8 LCL (Long et al., 2009) (both provided by Heather Long), LCL3 (Sinclair et al., 1994) (provided by Alison Sinclair) and IB4 cells (King et al., 1980) (provided by Martin Rowe) were established by infection of primary B cells with the B95-8 strain of EBV (type I). They were passaged 1:6 twice weekly using RPMI media supplemented with 10% FBS and 5% PSG.

### **2.1.1. Freezing cells**

200 mls of cells were pelleted after centrifugation and resuspended in 5 mls of freezing mix. Cells were aliquoted into 5 cryogenic vials (Nunc), and frozen at  $-80^{\circ}\text{C}$  in a container with

isopropanol (Nalgene). Vials were transferred to liquid nitrogen storage after at least 24 hrs at -80°C.

### **2.1.2. Thawing cells**

Cells were transferred from liquid nitrogen storage to a 37°C water bath. Once thawed, the 1 ml of cells were added to 10 mls pre-warmed RPMI media in a 25 ml flask and incubated overnight at 37°C with 5% CO<sub>2</sub>.

### **2.1.3. Haemocytometer cell counting**

15 µl of cells were counted using a Neubauer haemocytometer, which is divided into 9 equally spaced squares. Cells located in the 4 corners were counted, any cells outside or on these defined regions were not counted. The average cell counts of the four squares were used to calculate the cell culture concentration using the formula:

$$\text{Cells/ml} = \text{Average cell count} \times 1 \times 10^4 \text{ cells}$$

### **2.1.4. Cell proliferation assays**

Cells were diluted to  $2 \times 10^5$  or  $4 \times 10^5$  cells/ml and incubated overnight at 37°C with 5% CO<sub>2</sub>. Cells were then treated with DMSO, 75 nM, 150 nM or 300 nM Flavopiridol for 96 hrs. Upon addition of 1:1 Trypan blue (Sigma), cells that were not stained blue (alive) were counted every 24 hrs.

To test cell viability in Accell delivery media (Dharmacon) for NELF knockdown, Mutu III cells were set up at  $5 \times 10^5$  cells/ml and incubated overnight at 37°C with 5% CO<sub>2</sub>. Cells were pelleted and resuspended in either 1ml RPMI media, serum-free Accell media or Accell media containing 2% FBS in a 12 well plate (Starstedt). Cells were counted by diluting in 1:1 Trypan blue every 24 hrs for 72 hrs. RNA was targeted for knockdown using the Accell SMARTpool mixture containing 4 different siRNAs targeting NELF-A (WHSC2) or a non-targeting control (see Table 1). siRNAs were stored as 100 µM stocks in 5X siRNA buffer at -20°C.

siRNA	Target Sequence
1	GUUUUGUAUUCUUCUUUG
2	GUACCUAUUUUCAUUGUAA
3	CCGACAUCUUGAAGUCCUU
4	CCAACGUUCAGGAUAUUUU

Table 1

## 2.2. Biochemistry

All reagents were purchased from Fisher Scientific unless stated.

### 2.2.1. Reagents

#### EBC buffer

50 mM Tris-HCL pH 8.0, 120 mM NaCl, 0.5% NP-40, 5 mM DTT in sterile millipore water

#### ECL solutions I (1 ml)

125  $\mu$ M Luminol (250 mM stock in DMSO), 20  $\mu$ M coumaric acid (Sigma) (90 mM stock in DMSO) and 5 mM Tris pH 8.5 in 1 ml sterile millipore water

#### ECL solution II (1 ml)

0.0075% Hydrogen Peroxide ( $H_2O_2$ ) and 5 mM Tris pH 8.5 in 1 ml sterile millipore water.

#### Gel sample buffer

50 mM Tris, 4% SDS, 5% 2-Mercaptoethanol (Sigma), 10% Glycerol, 1 mM EDTA and 0.01% bromophenol blue

#### PBS-Tween

100 PBS tablets (Oxoid) and 10 mls Tween-20 made up in 10 L  $dH_2O$ .

### **Stripping buffer for western blots**

100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris-HCL pH 6.7.

### **Transfer buffer**

15 g Tris, 72 g Glycine, 4 L dH<sub>2</sub>O and 1 ml Methanol

### **2.2.2. Preparation of total cell lysates (TCL)**

Cells were washed in PBS, counted and resuspended in 100  $\mu$ l/ $1 \times 10^6$  cells of 1x GSB. Cells were sonicated on ice using the Vibra-Cell VC 750 sonicator (Sonics) for 7 pulses at 25% amplitude for 10 seconds with 10 second gaps. Samples were boiled at 95°C for 10 mins, vortexed, briefly centrifuged for 30 seconds and stored at -20°C.

### **2.2.3. Immunoprecipitation (IP)**

Mutu III cells were diluted to  $5 \times 10^5$  cells/ml and incubated overnight at 37°C. Cells were centrifuged and counted. Following a PBS wash,  $20 \times 10^6$  cells were lysed in 1 ml EBC buffer for 30 mins on ice and sonicated for 7 pulses at 25% amplitude for 10 seconds with 10 second gaps. Insoluble debris was pelleted by centrifugation at 13000rpm for 5 mins at 4°C. Cleared lysates were then pre-cleared for 1 hr using a 1:1 slurry mixture consisting of EBC buffer and either 80  $\mu$ l protein A or protein G sepharose beads (depending on antibody isotype). Beads were pelleted by centrifugation, for 1 min at 4000 rpm at 4°C. 2x 50  $\mu$ l samples were removed and frozen at -20°C at this point. Lysates were divided into 2 eppendorfs each containing 500  $\mu$ l per IP ( $1 \times 10^7$  cells), treated with 2  $\mu$ g CDK9, Cyclin T1 or Brd4 or 4  $\mu$ g (PE2) primary antibodies (see appendix A) and incubated with rotation for 3 hrs at 4°C. Immune complexes were collected using 40  $\mu$ l 1:1 protein A or protein G slurry and were incubated with rotation overnight at 4°C.

The following day samples were washed three times in EBC buffer and given a final wash in ice-cold PBS. After the final wash 20  $\mu$ l of 2x gel sample buffer was added to the beads and

immune-complexes were released by boiling for 10 mins at 95°C. 15 µl of 5x gel sample buffer was added to cell lysates samples and boiled for 10 mins at 95°C.

#### **2.2.4. SDS PAGE**

10-20 µl samples were loaded into pre-poured 10% Bis-Tris, 4-12% Bis-Tris or 3-8% Tris-acetate (Brd4 only) gels (Invitrogen) using a Hamilton syringe. Lysates were resolved using either 1x MOPS or 1x Tris acetate running buffers (Invitrogen) depending on the desired separation. 15 µl of SeeBlue Plus2 pre-stained standard marker (Invitrogen) was also loaded and electrophoresis carried out for 50-60 mins at 200 V

#### **2.2.5. Immunoblotting**

After separation by SDS-PAGE, proteins were transferred onto Protran nitrocellulose membranes (Whatman) in transfer buffer at 85 V for 90-120 mins using a blotting cell (Bio-Rad). Membranes were incubated with ponceau stain (Sigma) for one minute to examine loading and verify a successful transfer. Membranes were cut as required to probe for multiple proteins on one blot. Membranes were washed three times for 10 minutes in PBS-Tween on a shaker and blocked for 1 hr using 5% milk powder (Marvel) in PBS-Tween. Primary antibodies (Appendix C) were added to membranes in a 5% milk PBS-Tween solution and incubated overnight at 4°C with rocking. After further washing with PBS-Tween (3x10 mins), secondary antibodies (Appendix C) made up in 5% milk PBS-Tween solution conjugated to a horse-radish peroxidase (HRP) enzyme were added to membranes and incubated with rocking at room temperature for 1 hr. Final washing (3x10 mins) in PBS-Tween was performed and equal volumes of ECL solutions I & II were added to membranes. Membranes were exposed to Fuji medical X-ray film (Fisher) for varying time periods and developed using the Konica SRX-101A film processor. Blots were stored at 4°C for re-probing.

#### **2.2.6. Stripping gels**

In order to re-probe blots, membranes were washed with dH<sub>2</sub>O to remove any excess ECL solutions. Membranes were heated to 50°C for 15 to 20 mins in stripping buffer and washed 3



times in PBS-Tween for 5 mins. Membranes were then blocked using 5% milk PBS-Tween solution for 1 hr and probed with a different primary antibody.

### **2.2.7. *NELF A knockdown***

Prior to Accell delivery media addition (section 2.1.6), either 7.5  $\mu$ M non-targeting (NT) or NELF-A siRNA SMARTpool was added to a 24 well plate (Starstedt). 750  $\mu$ l of Mutu III cells at  $5 \times 10^5$  cells/ml in Accell delivery media were then added and incubated for 72 hrs at 37°C with 5% CO<sub>2</sub>. Cells were pelleted in 1 ml PBS for cell counting using Trypan blue.  $1 \times 10^5$  cells were processed for Relative Quantification (section 2.3.9) using the Power SYBR Green Cells-to-CT Kit (Applied Biosystems) according to the manufacturer's specifications. The remaining cells were resuspended in GSB and boiled at 95°C for 10 mins in preparation for SDS-PAGE (see section 2.2.4).

## 2.3. Molecular Biology

All solutions were made using sterile Millipore water

### 2.3.1. *Chromatin Immunoprecipitation (ChIP) buffers and reagents*

#### **Cell lysis buffer**

85 mM KCl, 0.5% NP-40, 5 mM PIPES pH 8.0, 1 mM PMSF (Sigma) and 1 EDTA-free protease inhibitor cocktail tablet (Roche).

#### **Elution Buffer**

10 mM Tris pH 8.0, 5 mM EDTA, 1% SDS

#### **High salt buffer**

0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 500 mM NaCl

#### **Immunoprecipitation dilution buffer**

0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris pH 8.0, 167 mM NaCl, 1 mM PMSF and 1 EDTA-free protease inhibitor cocktail tablet.

#### **Lithium Chloride buffer**

250 mM LiCl, 1% NP40, 1% Na deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.0

#### **Low salt buffer**

0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 150 mM NaCl

#### **Proteinase K (Sigma)**

Dissolved in water and stored as 20 mg/ml aliquots at -20°C

**SDS lysis buffer**

1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, 1 mM PMSF and 1 EDTA-free protease inhibitor cocktail tablet.

**TE buffer**

10 mM Tris pH 8.0, 1 mM EDTA

**2.3.2. *Bead preparation***

Protein sepharose A beads (Sigma) were pre-swollen overnight in PBS, washed twice with PBS and twice with IP dilution buffer or EBC buffer. 500 µl Protein sepharose A beads were blocked using 350 µg of single-stranded sonicated salmon testes DNA (Sigma) and were rotated for 1 hr at 4°C. Beads were stored as 1:1 slurry with IP dilution buffer at 4°C prior to use.

Protein sepharose G beads (Sigma) did not require pre-swelling or blocking. Instead they were washed twice with PBS, twice with IP dilution buffer and were stored in IP dilution buffer at 4°C prior to use.

**2.3.3. *Chromatin preparation***

Cells were resuspended to  $5 \times 10^5$  cells/ml 24 hrs prior to chromatin preparation. Cells were diluted to  $1 \times 10^7$  cells/ml in RPMI 1640 media, crosslinked using 1% formaldehyde (Sigma) for 15 mins on a rocker and cross-linking was halted by incubation with 125 mM glycine for 10 mins at room temperature. Cells were pelleted by centrifugation at 1300 rpm for 10 mins at 4°C and washed in PBS. Cell pellets were lysed on ice for 10 mins in 300 µl cell lysis buffer per  $10^7$  cells. Nuclei were pelleted at 8000 rpm for 5 mins at 4°C and resuspended in 200 µl SDS lysis buffer per  $1 \times 10^7$  cells. Chromatin was then sonicated on ice to sizes between 200 bp and 600 bp and stored as aliquots at -80°C.

Where required, cells were incubated with either 500 µM DRB (Sigma) for 2 hrs or 50 nM JQ1 (gift from Stefan Knapp) for 48 hours at 37°C. DMSO was used for control samples.

### ***2.3.4. Chromatin Immunoprecipitation (ChIP) normal method***

100  $\mu$ l ( $5 \times 10^6$  cells) of chromatin was diluted ten-fold in IP dilution buffer and pre-cleared using 22.5  $\mu$ l of unblocked protein A Sepharose beads at 4°C for an hour. An input control (40  $\mu$ l) was removed from the supernatant and stored at -20°C. The remaining chromatin was incubated with or without (no antibody control) primary antibodies (see Appendix A) overnight at 4°C with rotation. Secondary antibodies were added where required (Appendix A) and samples rotated for a further 2 hrs 4°C. Immune complexes were collected by rotating for 3 hrs at 4°C with 22.5  $\mu$ l of pre-blocked protein A sepharose beads. Samples were then washed for 10 mins with rotation at 4°C using each of the following wash buffers; 1x low salt, 1x high salt, 1x LiCl and 2x TE. Immune complexes were eluted in 150  $\mu$ l elution buffer at 65°C for 20 mins. Beads were removed and DNA-protein immuno complexes were incubated at 65°C overnight to reverse crosslinking. Input controls were also incubated overnight in 150  $\mu$ l elution buffer to reverse crosslinks. Proteinase K (50  $\mu$ g) and TE buffer (150  $\mu$ l) were added to samples and protein digested for 2 hrs at 50°C. DNA was purified using a QIAquick® Gel extraction kit (Qiagen) according to the manufacturer's instructions and eluted in 110  $\mu$ l sterile millipore water.

### ***2.3.5. Chromatin Immunoprecipitation (ChIP) pre-absorbed method***

In a variation to the normal ChIP protocol, for certain transcription factors (Appendix B), a mix of protein G sepharose beads (Sigma) and protein A sepharose beads were incubated with primary or secondary antibodies before chromatin was added. 50  $\mu$ l of 50:50 protein A and protein G sepharose beads were incubated with secondary antibodies in 500  $\mu$ l of IP dilution buffer. Alternatively, 1 ml of the rat hybridoma supernatants were used to pre-coat beads. After an overnight incubation with rotation at 4°C, the primary antibodies (Appendix B) or mouse hybridoma supernatants were incubated with rotation at 4°C for 3 hrs. 1 ml of pre-cleared chromatin was added to the beads and incubated overnight with rotation at 4°C. Samples were then processed as in section 2.3.4.

### **2.3.6. RNA isolation**

RNA was extracted from  $5 \times 10^6$  cells using 1 ml TRI Reagent (Sigma) and purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's guidelines.

### **2.3.7. Determining RNA concentration**

After purification, RNA concentrations were determined using an eppendorf BioPhotometer. Samples were diluted 1 in 50 (2  $\mu$ l in 98  $\mu$ l sterile Millipore water) in a UV-Cuvette micro (Brand) and RNA concentration ( $\mu$ g/ml) was calculated using the formula  $A_{260} \times 40 \mu\text{g/ml} \times \text{dilution factor}$  (50). The purity was measured by the ratio of the two absorbances between 260 nm and 280 nm (1.8 to 2 for pure RNA).

### **2.3.8. cDNA synthesis**

cDNA was synthesised from purified RNA using the ImProm-II Reverse Transcription system (Promega) according to the manufacturers specifications. 1  $\mu$ g of RNA was incubated at 70°C for 5 mins with 0.5  $\mu$ g of random primer and millipore sterile water in a final volume of 5  $\mu$ l. Samples were rapidly cooled on ice for 5 mins. For reverse transcription, samples were added to 15  $\mu$ l of master mix containing 4.5  $\mu$ l millipore sterile water, 4  $\mu$ l ImProm-II 5X Reaction Buffer, 4  $\mu$ l  $\text{MgCl}_2$ , 1  $\mu$ l dNTP mix, 0.5  $\mu$ l recombinant RNasin ribonuclease inhibitor and 1  $\mu$ l ImProm II reverse transcriptase per reaction. Primers were then annealed at 25°C for 5 mins, extended at 42°C for 60 mins, heated to 70°C for 15 mins. cDNA samples were stored at -80°C.

Alternatively cDNA was synthesised using the Power SYBR Green Cells-to-CT kit according to the manufacturer's instructions.

### **2.3.9. Q-PCR**

Quantitative PCR (Q-PCR) was performed using an Applied Biosystems 7500 real-time PCR machine. For ChIP analysis, 5  $\mu$ l DNA was added to a SYBR green master mix containing 12.5  $\mu$ l 2xSensiMix SYBR (Bioline), 150 nM forward and reverse primers (Appendix D) and sterile millipore water to a final volume of 25  $\mu$ l. Samples were heated to 95°C for 10 mins, followed by

40 cycles at 95°C for 15 sec and 60°C for 1 min and dissociation curve analysis. Input controls were serially diluted 4 fold to generate a standard curve for each primer set (Appendix D). Using the 7500 System SDS Software, the crossing threshold values ( $C_t$ ) for all samples were converted into percentage input values using the standard curves generated from the input controls.

For cDNA analysis, samples were either analyzed using the absolute quantification method or by relative quantification (ddCt). For absolute quantification, standard curves were generated from either Mutu I or Mutu III cDNA. Transcript levels were determined using Qp and Cp-specific primers or cDNA-specific CD23 and CD79B-specific primers with the 18S rRNA Quantitect primer assay (Qiagen) used as a normalization control (Appendix E). For relative quantification (ddCt), Q-PCR was carried out using Power SYBR Green Cells-to-CT kit (Applied Biosystems) to the manufacturer's specifications. Transcript levels were determined using cDNA-specific NELF, EBNA 2, EBNA 1 (YUK) or LMP1-specific primers (Bell et al., 2006) with actin as the endogenous control.

### **3. Investigating the regulation of CTD phosphorylation and transcriptional elongation at the key EBV latency promoter Cp**

The Epstein-Barr Virus latent protein EBNA 2 is required for the immortalisation and continued proliferation of EBV-infected B cells (Kempkes et al., 1995a, Cohen et al., 1989). Through interactions with cellular DNA binding proteins EBNA 2 binds upstream of 3 viral promoters, the C promoter (Cp), LMP 1p and LMPp 2A to regulate the expression of 9 viral genes in latency III cells (Sung et al., 1991, Ghosh and Kieff, 1990, Wang et al., 1990b, Jin and Speck, 1992, Fahraeus et al., 1990a). Through RBP-Jk binding sites, found upstream of LMP1p, EBNA 2 augments the expression of the short 2.8kb transcript encoding the membrane protein LMP1 (Wang et al., 1990b). LMP1 is not only essential for EBV transformation of B cells (Kaye et al., 1993), it potentiates growth and survival through the activation of the cellular NFκB and MAPK pathways (Paine et al., 1995, Mitchell and Sugden, 1995, Herrero et al., 1995, Huen et al., 1995, Roberts and Cooper, 1998). Through the viral C promoter, EBNA 2 regulates the transcription of a polycistronic message of 120 kb which is differentially spliced generating transcripts encoding the 6 latent nuclear proteins, 5 of which are required for immortalisation of B cells (Sung et al., 1991, Ghosh and Kieff, 1990, Wang et al., 1990b, Jin and Speck, 1992, Fahraeus et al., 1990a).

EBNA 2 can interact with members of both the GTFs and HAT complexes including; TFIIB and TAF40 and p300/CBP (Wang et al., 2000, Tong et al., 1995c). However the mechanistic action of EBNA 2 at viral promoters is still not fully understood. Previous work from our laboratory using a conditionally active EBNA 2 cell line (ER/EB2.5) (Kempkes et al., 1995a), demonstrated that EBNA 2 binding to Cp resulted in the recruitment of Pol II and subsequent phosphorylation on the CTD at serine 5 residues (Bark-Jones et al., 2006). However, the limitations in detecting many transcription factors using ChIP assays in ER/EB2.5 cells hampered further investigation. Interestingly, ChIP assays using both a BL cell line (Mutu I) and an LCL demonstrated a clear increase in ChIP signal for many histone modifications in BL cells, highlighting the potential for these cells for our further studies (Chau et al., 2006).

The Mutu I cell line is an EBV-positive BL cell line, derived from a tumour biopsy (Gregory et al., 1990). They express the viral latency I transcriptional programme, consisting of only the EBNA 1 protein derived from a Qp-initiated transcript, therefore Cp is switched off. However, in culture Mutu I cells can drift to express a latency III state, referred to as Mutu III. Unlike Mutu I cells, Mutu III cells express all 9 viral latent proteins including EBNA 2, with both C and LMP1 promoters active (Gregory et al., 1990). It was therefore possible to perform ChIP analysis in an EBNA 2 +/- system in the same cell background. Using ChIP assays we determined how EBNA 2 efficiently promoted long-range transcription from Cp to yield all the EBNAs required for immortalisation.

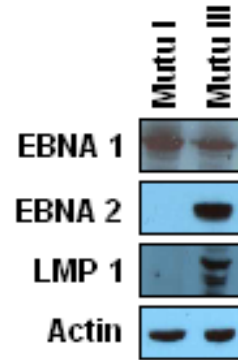
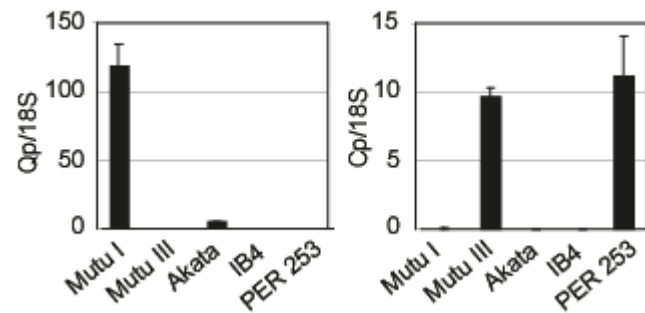
### **3.1. Validation of the Mutu I and Mutu III cell lines**

Prior to starting ChIP analysis, we confirmed that Mutu I (clone 179) and Mutu III (clone 48) (gifts from Professor Martin Rowe) cell lines were displaying the correct pattern of gene expression. Western blots were carried out using anti-EBNA 1, EBNA 2 and LMP1 antibodies (Figure 5A). Both cell lines expressed EBNA 1 protein and as expected only Mutu III cells expressed both EBNA 2 and LMP1. To confirm EBNA 1 derived from Qp in Mutu I cells and not the latency III promoter Cp, Qp and Cp-specific transcripts were analysed from Mutu cell-lines and control EBV positive cell lines (Figure 5B). Large quantities of Qp transcripts were detectable in Mutu I and the latency I cell line Akata. As expected, no Qp transcripts were detected in the latency III cells, Mutu III, IB4 or PER 253 B95-8 LCL. Conversely, no Cp transcripts were detectable in Mutu I, Akata or the IB4 LCL where Cp is inactive. Cp transcripts were detectable in both Mutu III and PER 253 cells. These data therefore confirm that Mutu I and Mutu III cells were displaying the correct pattern of gene expression.

### **3.2. EBNA 2-dependent Pol II serine 2 and serine 5 CTD phosphorylation detected at C promoter proximal and distal regions**

Firstly we set out to determine whether EBNA 2 expressed in Mutu III cells did in fact bind upstream of Cp. Our ChIP assays confirmed that EBNA 2 binding to Cp peaked around the



**A****B**

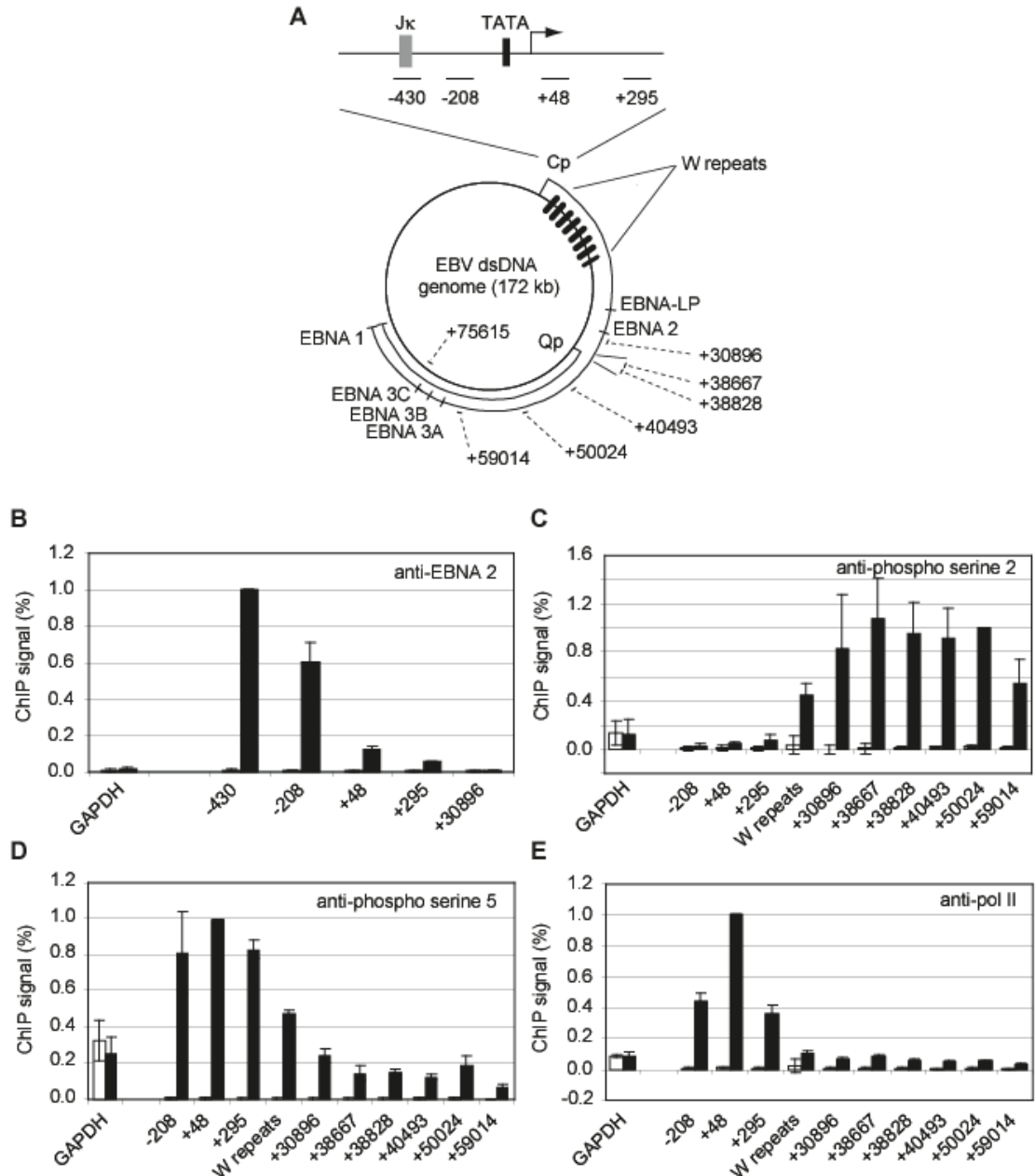
**Figure 5. Validation of Mutu I and Mutu III cells.** (A) Western blot analysis of whole cell lysates of Mutu I and Mutu III cells. Blots were probed with M.S. human serum to detect EBNA 1, PE2 to detect EBNA 2, CS1-4 to detect LMP1 and re-probed with anti-actin antibodies as a loading control. (B) PCR amplification of Qp-specific and Cp-specific transcripts. Akata cells (Qp only) and the PER 253 LCL (Cp only) served as positive controls for Qp and Cp usage respectively. The IB4 LCL has a deletion upstream of Cp so is negative for Qp and Cp transcripts. Qp or Cp signals were normalised to 18S rRNA primer signals. Results show the mean  $\pm$  standard deviation of a representative experiment carried out in duplicate.

RBP-Jk site in Mutu III cells and was undetectable in Mutu I cells (Figure 6A and B), as observed in ER/EB2.5 cells (Alazard et al., 2003, Bark-Jones et al., 2006), EBNA 2-activated transcription in Mutu III cells resulted in increased Pol II serine 2 CTD phosphorylation from +295, increasing significantly in the W repeat region and remaining high up to approximately 60kb downstream (Figure 6C).

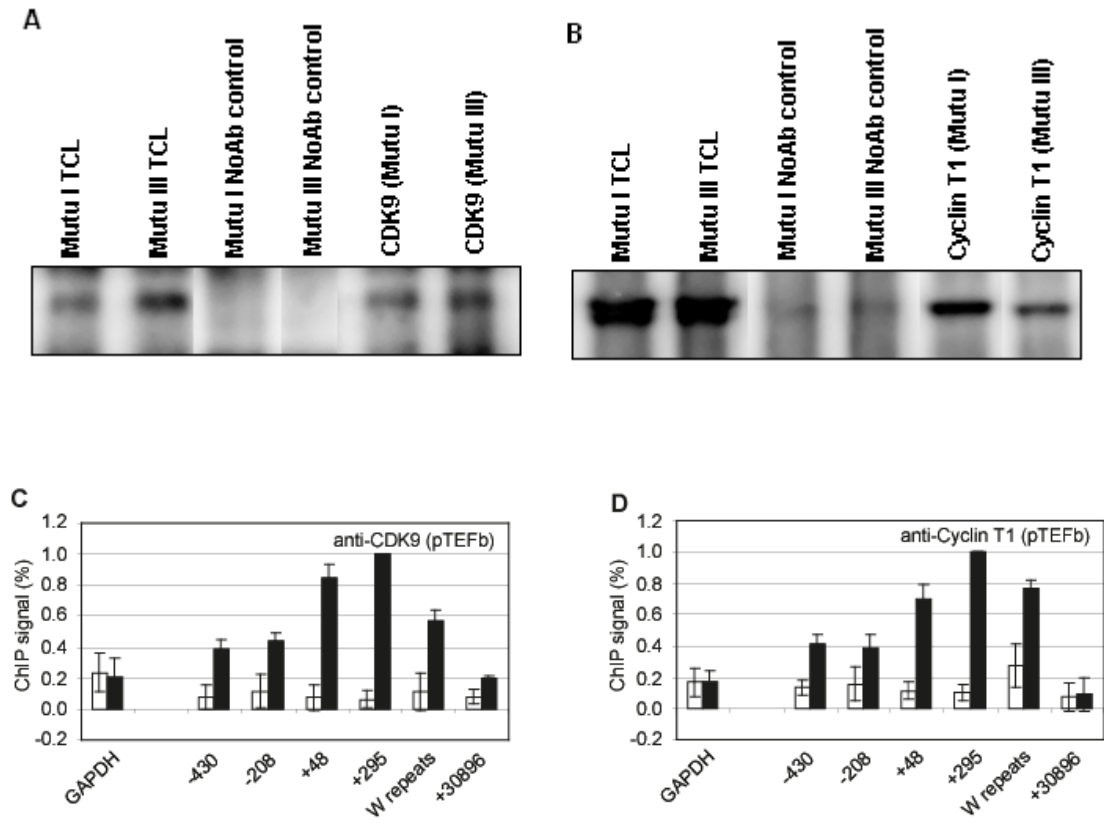
In agreement with our previous observations, we found that EBNA 2-activated Cp transcription resulted in large increases in Pol II recruitment and serine 5 CTD phosphorylation at promoter-proximal regions consistent with the promotion of transcription initiation (Figures 6D and E). We also found that increased serine 5 CTD phosphorylation was maintained at distal regions (Figure 6D). ChIP assays precipitating total Pol II detected increases in the association of Pol II with distal regions in Mutu III cells compared to Mutu I cells (Figure 6E), consistent with the promotion of transcriptional elongation by EBNA 2. Importantly, the observed changes in distal Pol II CTD phosphorylation could not be accounted for by increased Pol II presence alone, since increases in phospho-epitope levels exceeded the increases in total Pol II (Figure 6E).

### **3.3. EBNA 2-dependent pTEFb recruitment to Cp**

The cyclin dependent kinases CDK7, CDK8 and CDK9 have all been shown to phosphorylate the CTD of Pol II in order to regulate and enhance transcription (Komarnitsky et al., 2000, Lu et al., 1992, Hengartner et al., 1998, Borggreffe et al., 2002, Marshall et al., 1996). The phosphorylation of CTD serine residues acts as a scaffold to recruit transcriptional machinery such as the mRNA capping enzyme and elongation factors such as chromatin remodellers and splicing apparatus (Cho et al., 1997, Mccracken et al., 1997, Kim et al., 1997, Misteli and Spector, 1999, Wu et al., 2000b). We have previously demonstrated that EBNA 2-activated transcription requires pTEFb activity (Bark-Jones et al., 2006). Since CDK9 predominantly phosphorylates Pol II on serine 2 residues during elongation (Kim et al., 2002), we examined pTEFb recruitment at Cp. Initial western blotting analysis of ChIP samples confirmed that both pTEFb components could be immunoprecipitated from Mutu III cells (Figures 7A and B).



**Figure 6. High-level Pol II accumulation at Cp and CTD phosphorylation at distal EBV genome regions.** (A) Diagram showing the locations of the amplicons generated by the indicated primer sets (Appendix D) at the C promoter and around the circular EBV episome. Numbers indicate the 5' end of the forward primer relative to the Cp transcription start site in the annotated BP5-8 EBV sequence (NC\_007605.1). The RBP-J $\kappa$  site (grey box) and TATA box (black box) are shown. Percentage input signals, after subtraction of no antibody controls, are expressed relative to the highest signal obtained in all or the majority of experiments. ChIP in Mutu I cells (open bars) and Mutu III cells (black bars) using anti-EBNA 2 antibodies (n=3, c=2) (B), anti-phospho serine 2 Pol II CTD antibodies (n=3, c=3) (C), anti-phospho serine 5 Pol II CTD antibodies (n=3, c=2) (D) and anti-Pol II antibodies (n=3, c=2) (E). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.

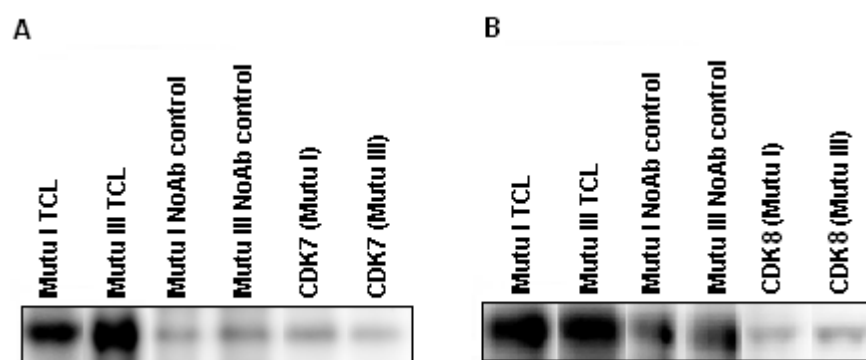


**Figure 7. pTEFb is recruited to Cp in Mutu III cells.** Western blotting of ChIPs using (A) CDK9 and (B) Cyclin T1 antibodies in Mutu I and Mutu III cells. Total cell lysates were used as positive controls for each immunoprecipitation. (C) and (D) percentage input signals, after subtraction of no antibody controls, are expressed relative to the highest signal obtained. ChIP in Mutu I cells (open bars) and Mutu III cells (black bars) using (C) anti-CDK9 antibodies (n=3, c=2) and (D) anti-Cyclin T1 antibodies (n=3, c=2). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.

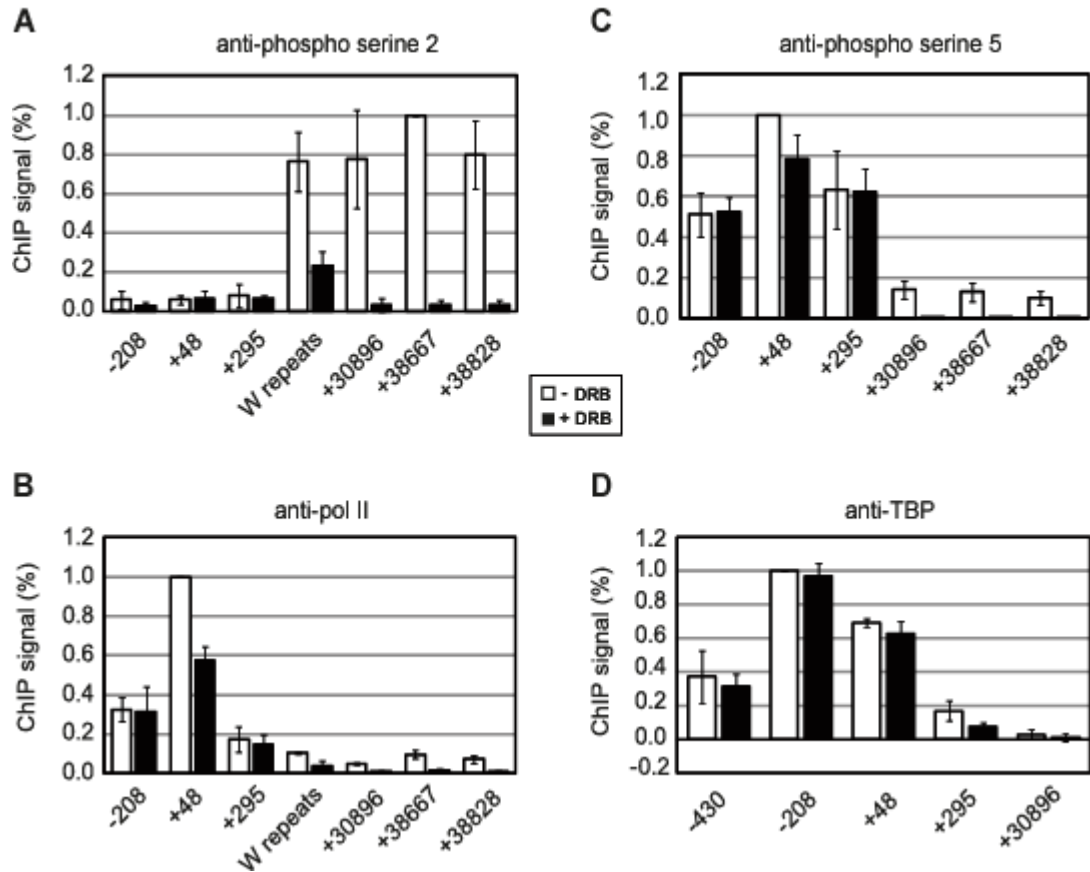
Next ChIP assays using anti-CDK9 and anti-Cyclin T1 antibodies demonstrated that high levels of both subunits of pTEFb were associated with Cp in Mutu III cells (Figures 7C and D). Consistent with a role for pTEFb in distal serine 2 CTD phosphorylation, pTEFb was also detectable in the W repeats (Figure 7) but fell to levels beyond the limits of detection of our ChIP assays thereafter. Previous studies have shown that pTEFb levels can drop significantly using standard ChIP methods even 2 kb downstream from promoters, despite clear evidence of pTEFb function (i.e. serine 2 phosphorylation) at these regions (Fujita et al., 2008, Egloff et al., 2009).

To investigate whether other CTD kinases were also present at Cp in Mutu III cells, we carried out ChIP assays for CDK7 and CDK8. However, western blotting of ChIP samples demonstrated that neither CDK7 nor CDK8 could be immunoprecipitated from Mutu III cells (Figures 8A and B). Further ChIP assays using secondary antibodies in conjunction with different ratios of protein sepharose A and G beads and varying primary antibody concentrations failed to immunoprecipitate either CDK7 or CDK8 protein (data not shown).

To further confirm that CDK9 was responsible for Pol II serine 2 CTD phosphorylation beyond 31 kb, Mutu III cells were treated with 500  $\mu$ M DRB for 2 hrs (Figure 9). DRB is a known inhibitor of CDK9 kinase activity, acting as an ATP analogue (Marshall et al., 1996). Our results demonstrated that DRB ablated Pol II serine 2 CTD phosphorylation and severely reduced polymerase retention at distal regions (Figure 9A and B). We also observed a reduction in Pol II serine 5 CTD phosphorylation at distal regions (Figure 9C). ChIP for TATA box binding protein (TBP) confirmed that DRB treatment did not have general non-specific effects on Cp pre-initiation complex assembly (Figure 9D). These data indicate that pTEFb is recruited to Cp in an EBNA 2-dependent manner, augmenting long-range transcription through Pol II CTD phosphorylation to transcribe all the EBNAs required for immortalisation.



**Figure 8. CDK7 and CDK8 do not immunoprecipitate in Mutu III cells under ChIP conditions.** Western blotting of ChIP samples using (A) CDK7 and (B) CDK8 antibodies after immunoprecipitation from Mutu I and Mutu III cells.



**Figure 9. The pTEFb inhibitor DRB reduces Pol II phosphorylation and retention in the Cp transcription unit.** ChIP carried out in Mutu III cells minus (open bars) or plus 500  $\mu$ M DRB (black bars) using anti-phospho serine 2 Pol II CTD antibodies (n=4, c=2) (A), anti-Pol II antibodies (n=4, c=3) (B), anti-phospho serine 5 Pol II CTD antibodies (n=4, c=2) (C) and anti-p300 antibodies (n=3, c=2) (D). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.

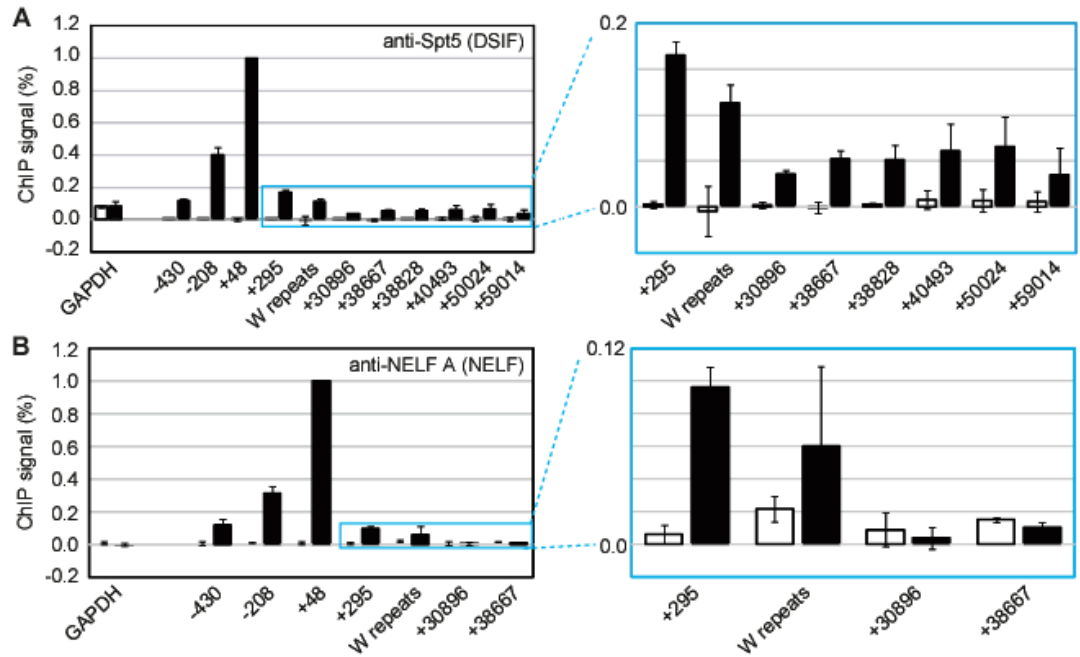
### **3.4. High-levels of the pausing complexes NELF and DSIF are present at Cp**

The high level of Pol II at Cp may be an indication of promoter-proximal pausing. We therefore examined the association of the pTEFb-regulated pausing complexes DSIF and NELF with Cp. DSIF and NELF induce promoter-proximal pausing that is relieved following the phosphorylation of DSIF, NELF and the Pol II CTD by pTEFb (Wada et al., 1998b, Ivanov et al., 2000, Bourgeois et al., 2002, Fujinaga et al., 2004). Once phosphorylated, NELF is released from the pausing complex. Conversely DSIF remains bound to the elongating Pol II complex and functions as a positive elongation factor (Wada et al., 1998a, Bourgeois et al., 2002). We detected high-levels of both the NELF-A subunit of the NELF complex and the Spt5 subunit of the DSIF heterodimer at Cp in Mutu III cells (Figure 10). Unlike NELF, which was absent from distal regions of the template, Spt5 remained detectable at distal regions consistent with a role for DSIF in promoting transcriptional elongation (Wada et al., 1998a, Bourgeois et al., 2002). Our results suggest that recruitment of pTEFb to Cp is likely to be required to overcome the high-level Pol II accumulation induced by DSIF and NELF, to promote elongation to distal regions through Pol II serine 2 CTD phosphorylation.

### **3.5. Low-level Pol II CTD serine 2 phosphorylation and pTEFb, NELF and DSIF recruitment at the EBNA 2-regulated LMP gene locus**

To determine whether polymerase stalling, high-level pTEFb recruitment and large increases in Pol II serine 2 CTD phosphorylation were evident at other shorter EBNA 2-responsive transcription units, we performed ChIP assays using primers specific for the EBNA 2-activated LMP genes. The EBNA 2-dependent LMP1, LMP2A and LMP2B genes encode transcripts of 2.8 kb, 11.7 and 8.4 kb in length (in the B95-8 EBV genome sequence NC\_007605.1). The LMP2A gene is located on the EBV positive strand, where transcription is regulated by EBNA 2 via two RBP-Jk sites (Laux et al., 1994b, Zimmer-Strobl et al., 1991). Conversely, EBNA 2-dependent LMP1 transcription is driven by a bi-directional promoter located in the reverse orientation in the EBV genome and activated via EBNA 2 binding to both RBP-Jk and PU.1 upstream sites (Johannsen et al., 1995). This bi-directional promoter also drives transcription of





**Figure 10 DSIF and NELF are recruited to Cp in Mutu III cells.** ChIP assays carried out in Mutu I (open bars) and Mutu III cells (black bars) using anti-Spt5 antibodies to detect DSIF (n=3, c=2) (A) or anti-NELF-A antibodies to detect NELF (n=3, c=3) (B). Blue-edged graphs show zoomed-in sections to allow better visualization of downstream primer signals. Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.

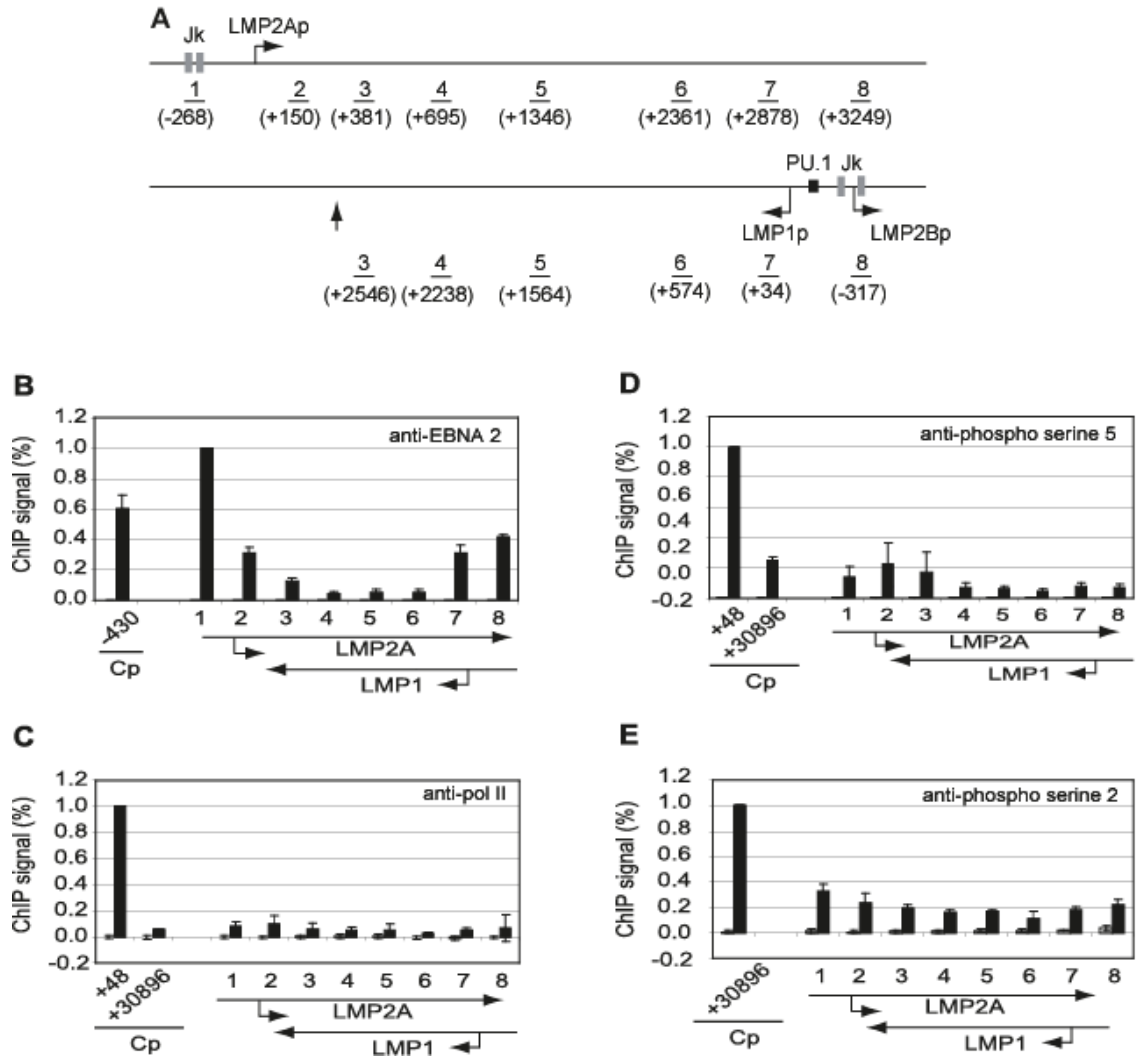
the LMP2B gene. The LMP2A and LMP1 transcription units therefore overlap and ChIP assays with primer sets 3-8 detect transcription complexes associated with either or both genes (Figure 11A). ChIP assays detected the same or higher levels of EBNA 2 binding to the LMP1 and LMP2A promoters in Mutu III cells to those detected at Cp (Figure 11B). However, no Pol II stalling was evident at either LMP promoter (Figure 11C). Consistently, Pol II serine 2 CTD phosphorylation did not reach the high levels observed at distal Cp regions and Pol II serine 5 CTD phosphorylation was also much reduced (Figures 11D and E). pTEFb recruitment to the LMP promoters was also barely detectable and NELF and DSIF recruitment to the LMP locus was also minimal (Figures 12A-E). Further experiments comparing both Cp and the LMP locus in an EBV-infected LCL confirmed our observations in Mutu III cells (Figure 13).

To exclude the possibility that low-level Pol II and transcription factor association with the LMP gene locus simply reflected low-levels of LMP transcription in the cell-lines under study, we used real-time PCR to determine the levels of Cp-initiated EBNA 2 and EBNA 1 transcripts and LMP1 transcripts in Mutu III cells and two EBV-infected LCLs (Figure 14). Although quantification of these transcripts is not directly comparable, we found that each cell line expressed similar levels of the EBV latent transcripts with variations in the level of transcripts produced between cell-lines probably as a result of differences in EBV genome copy number.

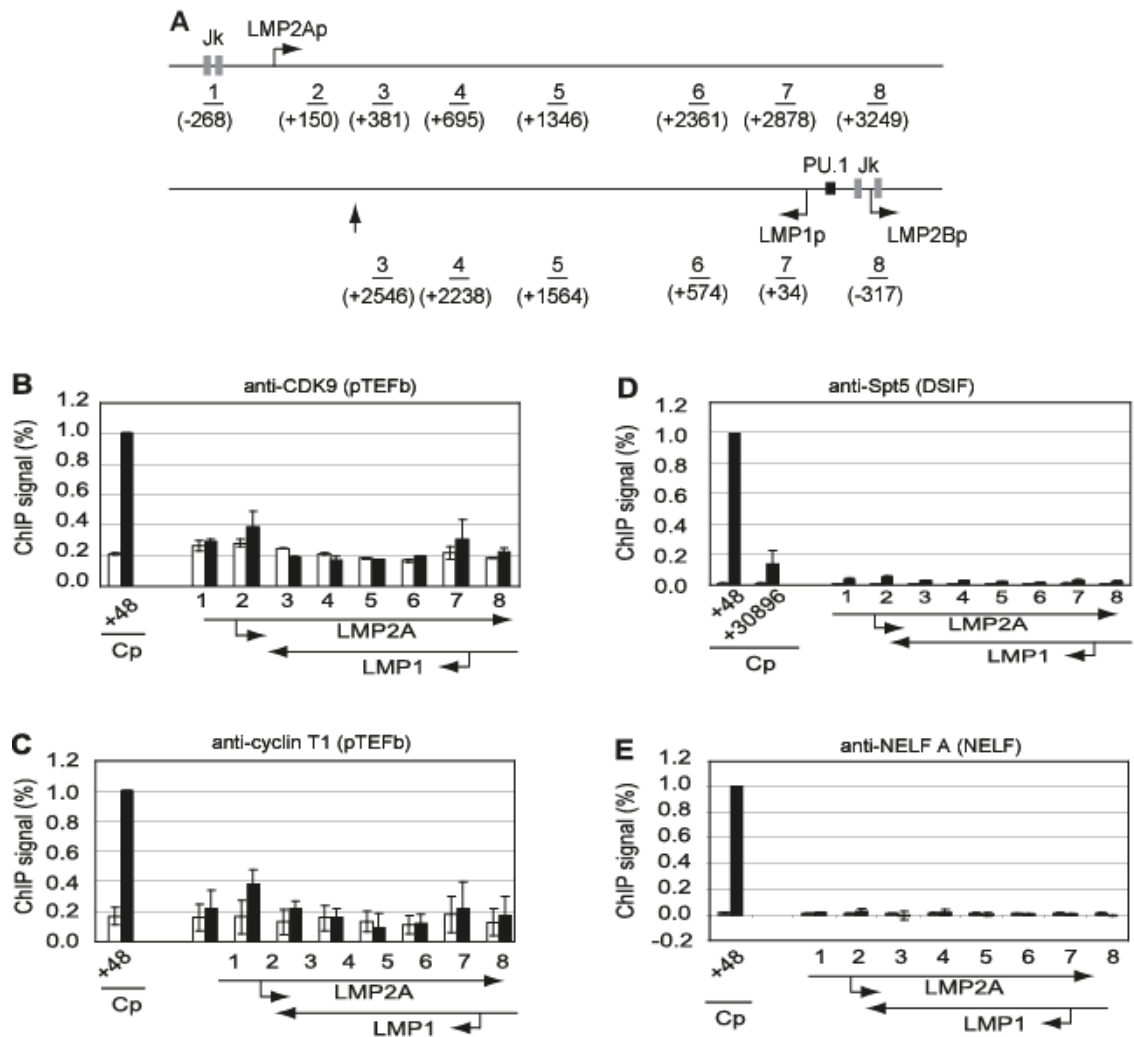
Taken together, our data indicate that Pol II accumulation and high-level pTEFb recruitment is not a general characteristic of EBNA2- activated promoters, but is specific to Cp. Moreover, the level of promoter-associated Pol II does not simply reflect the level of gene transcription from Cp and LMP1p.

### **3.6. Pol II stalling at Cp maintains a nucleosome-depleted region**

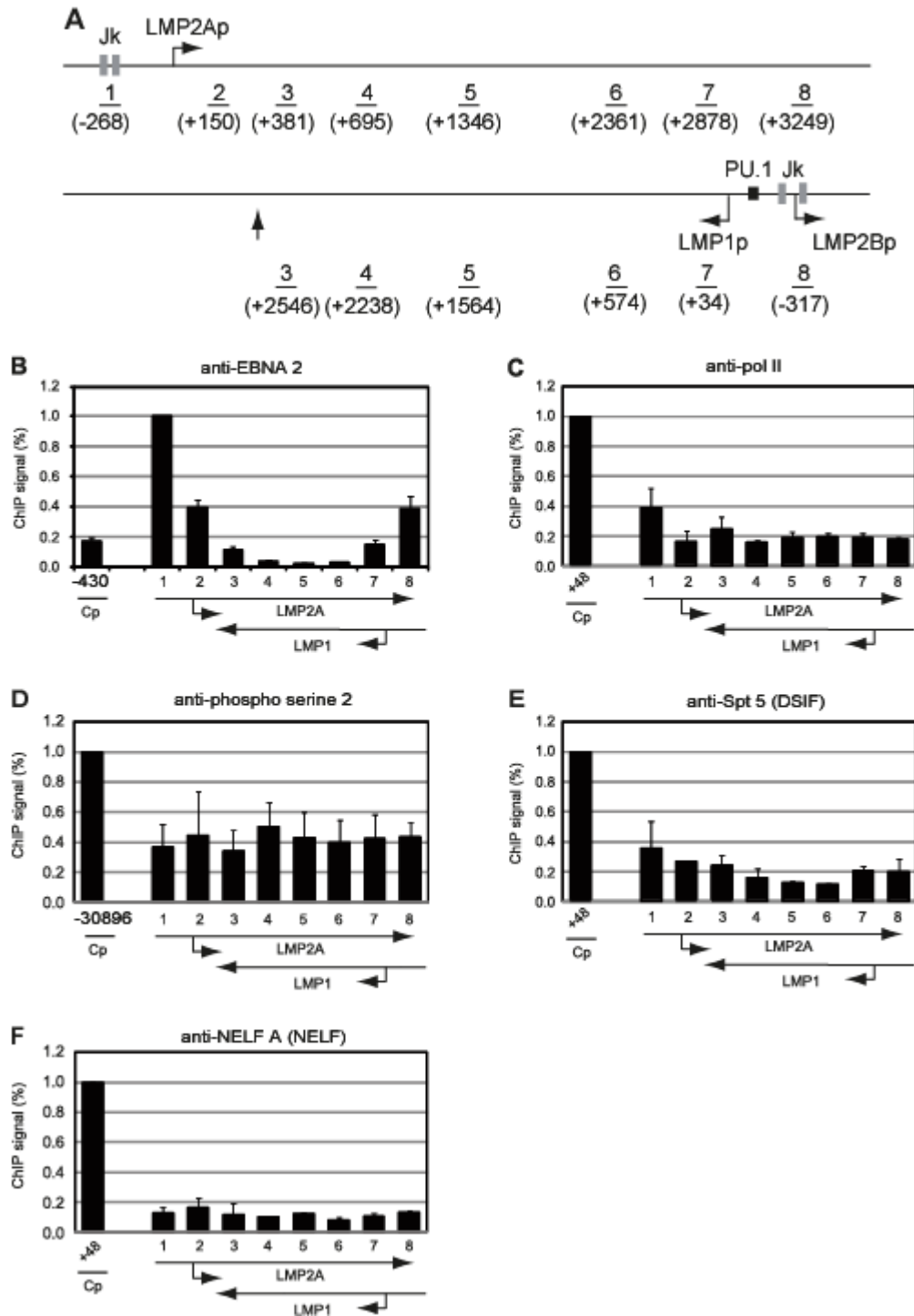
Pol II stalling has recently been implicated in the promotion of gene activity through the maintenance of a promoter-proximal nucleosome-free region (Gilchrist et al., 2010, Gilchrist et al., 2008). Therefore we investigated whether the region surrounding Cp was depleted of nucleosomes in Mutu III cells and an LCL where Cp is active, compared to Mutu I cells where Cp is inactive. Nucleosome levels were measured in ChIP assays using antibodies against the core histone, histone H3 (Egloff et al., 2009). Strikingly, we detected an 84% decrease in



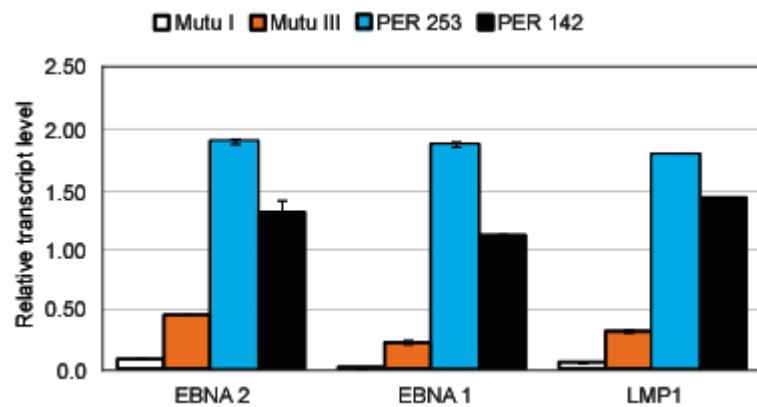
**Figure 11. Pol II is not paused at the LMP gene loci.** (A) Diagram of the LMP gene locus showing the locations of the amplicons generated by the indicated primer sets (1-8). Numbers indicate the 5' end of the forward primer relative to the starts of the LMP1 or LMP2A mRNA sequences (bent arrows) in the B95-8 annotated EBV sequence (NC\_007605). The RBP-J $\kappa$  sites (grey boxes) and PU.1 site (black box) are shown. The vertical arrow indicates the position of the LMP1 polyadenylation site. Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using Cp-specific primers with Mutu I (open bars) and Mutu III cell chromatin (black bars). ChIP using anti-EBNA 2 antibodies (n=3, c=2) (B), anti-Pol II antibodies (n=3, c=2) (C), anti-serine 5 Pol II CTD antibodies (n=3, c=2) (D) and anti-serine 2 Pol II CTD antibodies (n=3, c=3) (E). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.



**Figure 12. Elongation factors pTEFb, DSIF and NELF are not recruited to the LMP gene loci (A)** Diagram of the LMP gene locus showing the locations of the amplicons generated by the indicated primer sets (1-8). Numbers indicate the 5' end of the forward primer relative to the starts of the LMP1 or LMP2A mRNA sequences (bent arrows) in the B95-8 annotated EBV sequence (NC\_007605). The RBP-Jκ sites (grey boxes) and PU.1 site (black box) are shown. The vertical arrow indicates the position of the LMP1 polyadenylation site. Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using Cp-specific primers with Mutu I (open bars) and Mutu III cell chromatin (black bars). ChIP using anti-CDK9 antibodies (n=3, c=2) (B), anti-Cyclin T1 antibodies (n=3, c=2) (C), anti-Spt5 antibodies (n=5, c=2) (D) and anti-NELF antibodies (n=3, c=2) (E). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.



**Figure 13. Low level Pol II, DSIF and NELF recruitment at the LMP gene locus in an EBV immortalized LCL (PER 253 B95-8 LCL).** (A) Diagram of the LMP gene locus showing the locations of the amplicons generated by the indicated primer sets (1-8). Numbers indicate the 5' end of the forward primer relative to the starts of the LMP1 or LMP2A mRNA sequences (bent arrows) in the B95-8 annotated EBV sequence (NC\_007605). The RBP-Jk sites (grey boxes) and PU.1 site (black box) are shown. The vertical arrow indicates the position of the LMP1 polyadenylation site. Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using Cp-specific primers in an EBV immortalized LCL (PER 253 B95-8 LCL). ChIP using anti-EBNA 2 antibodies (n=6, c=2) (B), anti-Pol II antibodies (n=2, c=2) (C), anti-serine 2 Pol II CTD antibodies (n=5, c=2) (D) anti-Spt5 antibodies (n=2, c=2) (E) and anti-NELF antibodies (n=2, c=2) (F). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.



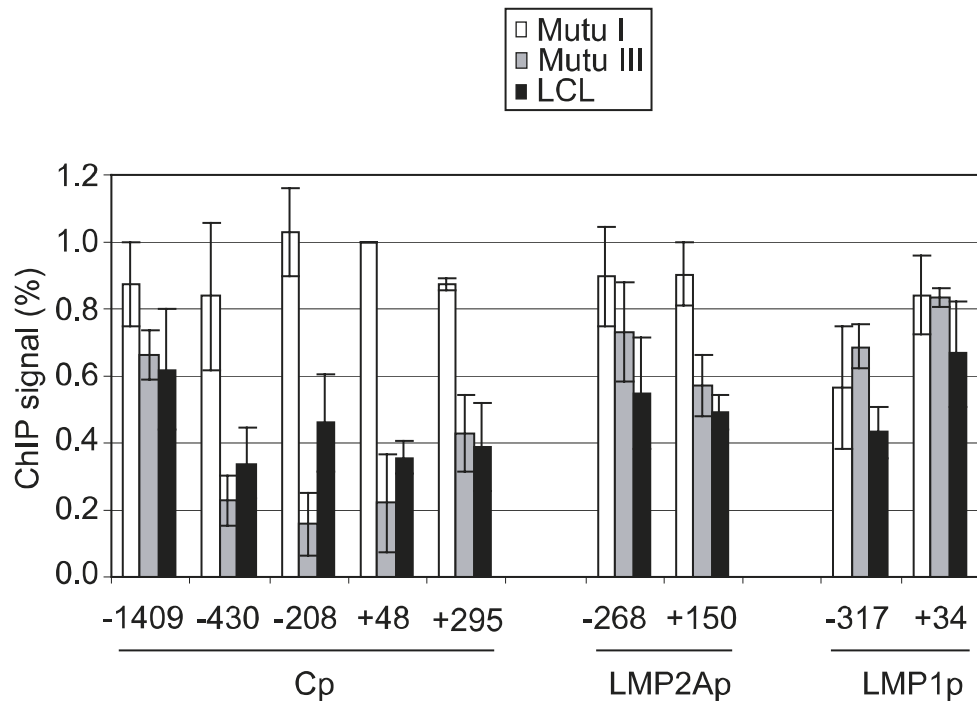
**Figure 14. Cp and LMP transcript levels are comparable in latency III cells.** Transcript levels were determined using cDNA prepared in parallel from Mutu I, Mutu III, PER 253 B95.8 LCL and PER 142 B95.8 LCL using exon-spanning Q-PCR primers (Appendix E) to detect EBNA 2, Cp-initiated EBNA 1 (YUK spliced) and LMP1 divided by actin quantities as a normalization control. Results show mean  $\pm$  standard deviation of Q-PCR duplicates from a representative experiment. Note that EBNA 1 transcripts initiate from Qp in Mutu I cells and are not detected by the YUK EBNA 1 primer set used here.

nucleosome occupancy at Cp in Mutu III cells compared to Mutu I cells using primer sets that spanned the region -208 to -96 upstream of the transcription start site and subsequent 78% and 73% decreases with primer sets spanning regions +48 to +167 and -430 to -337, respectively (Figure 15). Nucleosomes were similarly depleted from these regions in an EBV-infected LCL (Figure 15). In contrast, levels of nucleosome depletion at similar regions around LMP2Ap and LMP1p were much lower, consistent with the absence of stalled Pol II at these promoters (Figure 15). Therefore in the absence of Cp activity in Mutu I cells, nucleosomes assemble over promoter regions, but in the presence of stalled polymerase in Mutu III cells, Cp is maintained in a nucleosome-depleted state. In contrast, the low levels of Pol II initiating at the LMP promoters are unable to maintain a highly nucleosome-depleted region.

### **3.7. Pol II stalling at Cp may be directed by specific sequences that allow increased access to the transcription machinery**

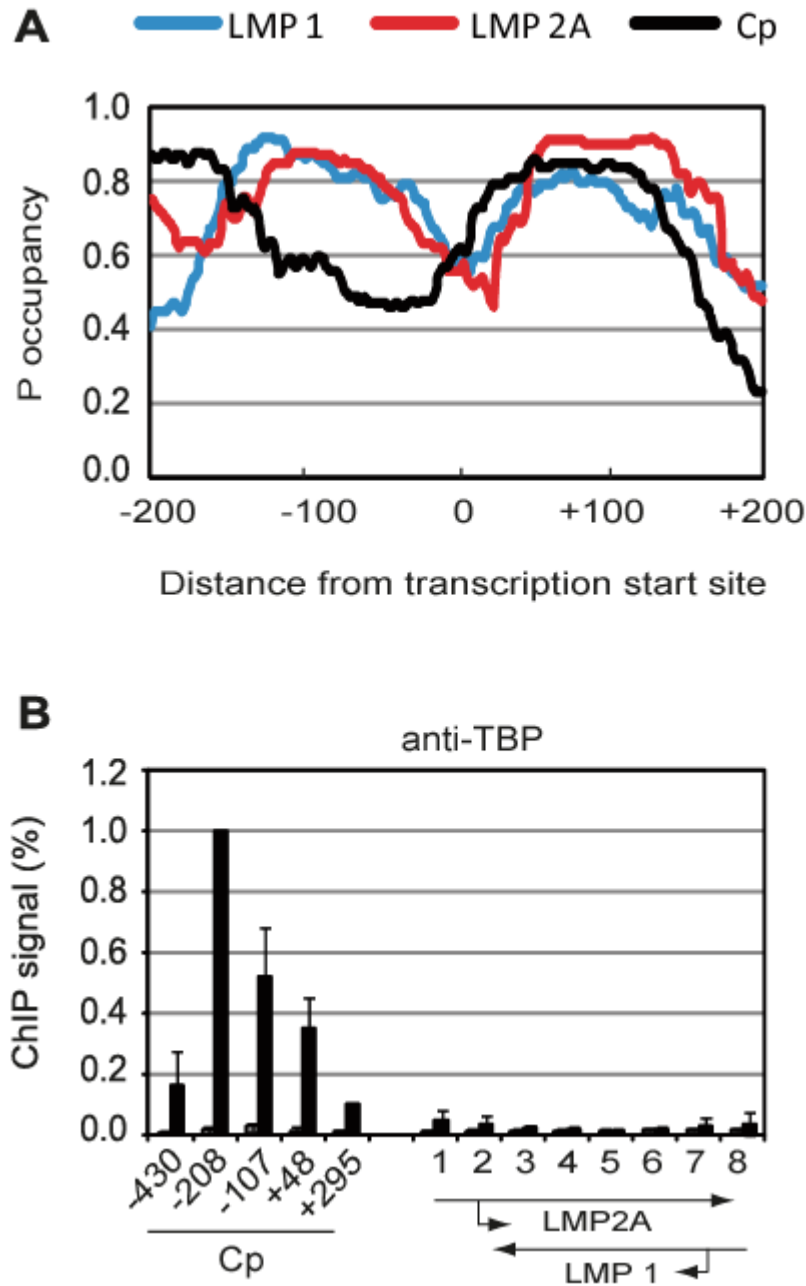
The active transcription of some protein-encoding genes relies on the ability of promoters to efficiently recruit Pol II through PIC assembly initiated by the binding of the GTF, TFIID. Therefore promoters containing sequences less favourable for nucleosome assembly could accumulate more stalled Pol II in conjunction with NELF and DSIF through increased access for initial TFIID binding. Using a nucleosome occupancy prediction programme [http://genie.weizmann.ac.il/software/nucleo\\_prediction.html](http://genie.weizmann.ac.il/software/nucleo_prediction.html) (Kaplan et al., 2009) we examined the likelihood of the DNA sequences around Cp to assemble into nucleosomes. This revealed a dramatic difference in the probability of nucleosome occupancy at Cp compared to the LMP promoters (Figure 16A). The sequences around the TATA box (-31 to -26) at Cp (black line) has a predicted lower propensity to accommodate nucleosomes when compared to TATA boxes (-32 to -27 and -28 to -23) at the LMP1 and LMP2A promoters (blue and red lines).

Further ChIP experiments using antibodies against the TFIID subunit TBP, detected lower level binding at the LMP promoters compared to Cp (Figure 16B). The TBP signal at Cp was maximal in the -208 to -96 region indicating the crosslinking of TFIID complexes to other transcription factors around Cp. Our data suggest that initial high-level recruitment of Pol II to Cp, presumably in association with the Pol II binding factors NELF and DSIF, is driven by increased accessibility of the promoter to TFIID. However, in the absence of



**Figure 15. Pol II pausing at Cp prevents nucleosome assembly around the promoter.** ChIP using anti-histone H3 antibodies. Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments carried out using at least 2 chromatin batches in Mutu I cells (open bars), Mutu III cells (grey bars) and the PER 253 B95-8 LCL (black bars). Primer sets probed promoter-proximal regions of Cp, LMP2Ap and LMP1p. Percentage input signals, after subtraction of no antibody controls are expressed for comparison purposes relative to the highest signal obtained.





**Figure 16. Pol II pausing at Cp is driven by DNA sequences that promote access by TBP.** (A) The probability of nucleosome occupancy (P occupancy) at regions upstream and downstream from the transcription start site (TSS) of Cp (thick black line), LMP1p (thick grey line) and LMP2Ap (thin black line) predicted using tools available at [http://genie.weizmann.ac.il/software/nucleo\\_prediction.html](http://genie.weizmann.ac.il/software/nucleo_prediction.html) (Kaplan et al., 2009). (B) ChIP using anti-TBP antibodies analysed using Cp and LMP gene primers (see Figure 11) in Mutu I cells (open bars) and Mutu III cells (black bars). Results show the mean  $\pm$  standard deviation of three independent experiments carried out using 2 chromatin batches. Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained at Cp.

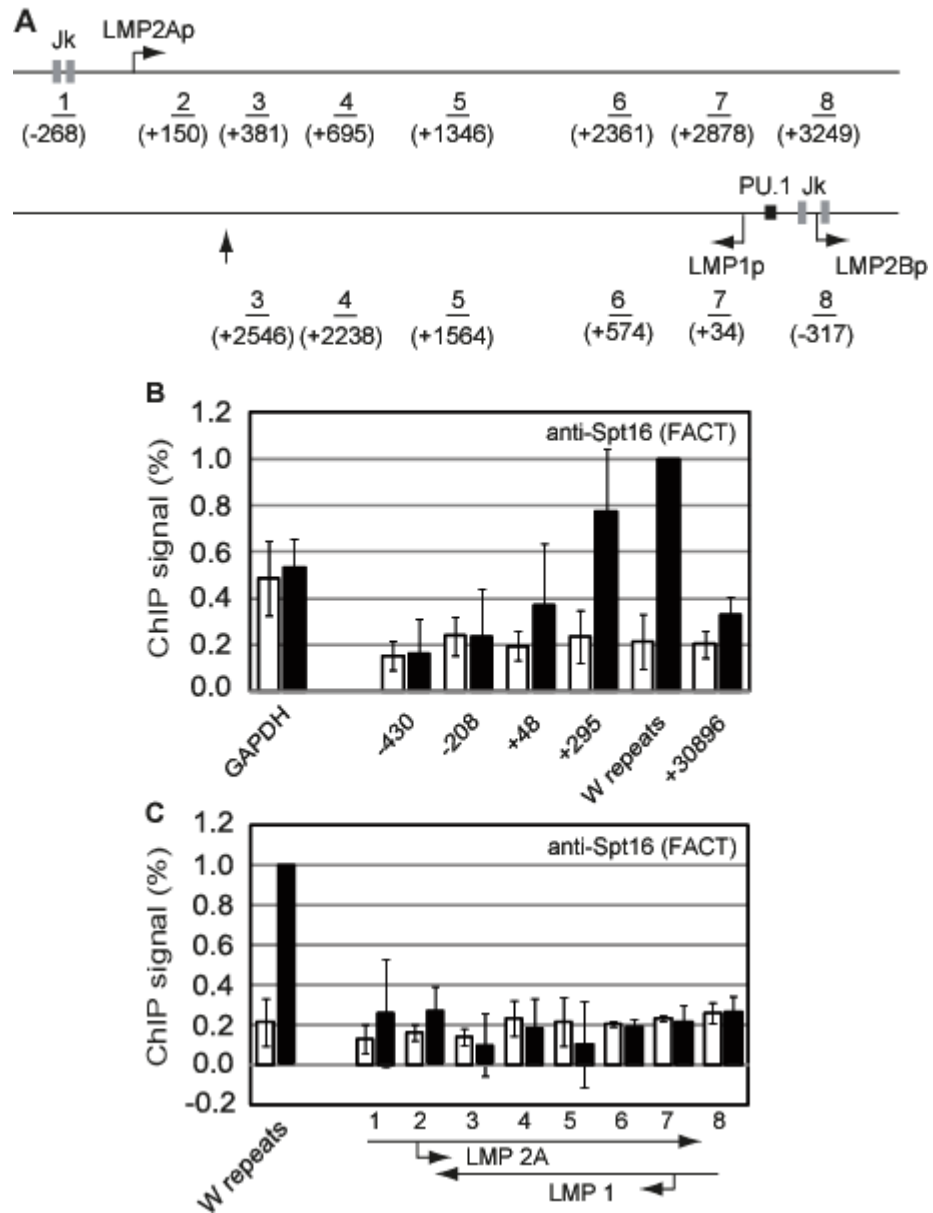
active Cp transcription in Mutu I cells, nucleosomes can assemble at Cp (Figure 15) and that the reduced probability of nucleosome occupancy may provide an initial advantage to pre-initiation complex assembly, but does not completely preclude nucleosome assembly.

### **3.8. FACT is recruited to aid transcription through Wp**

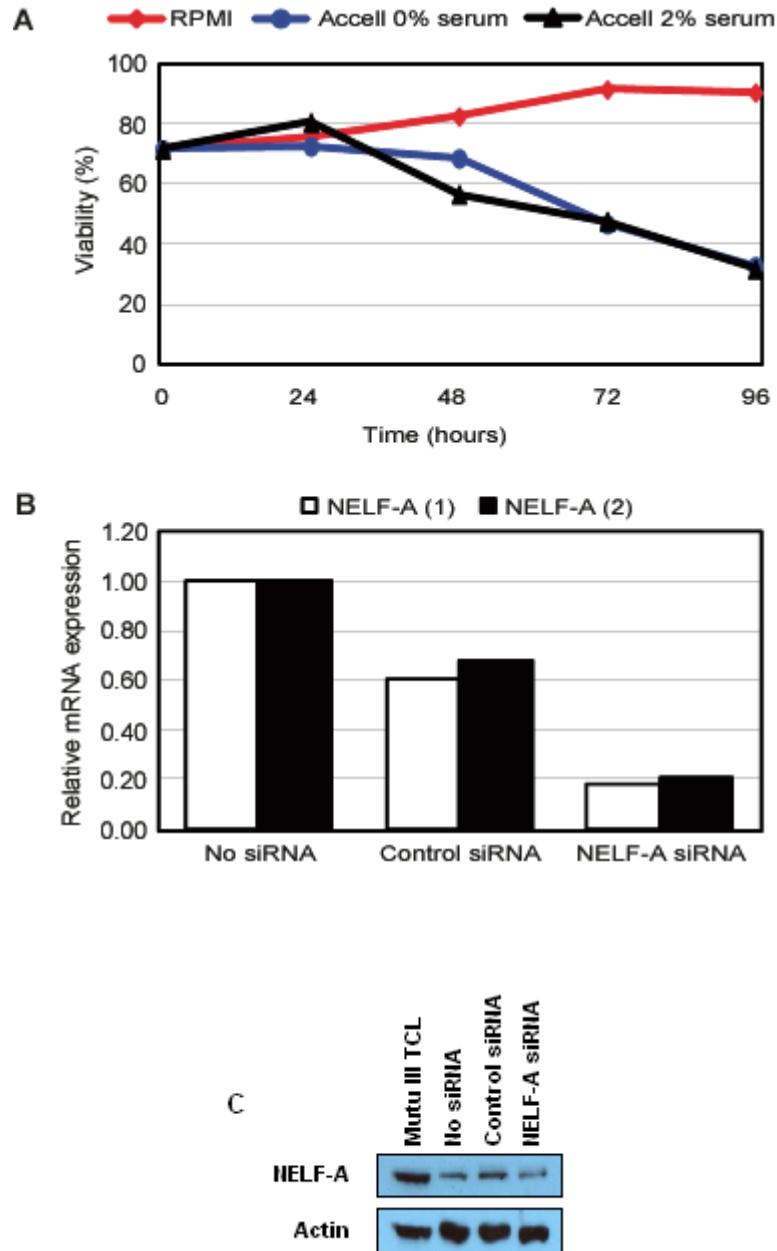
Elongating Pol II is required to overcome the obstacle of chromatinized DNA. To traverse nucleosomes successfully, the temporary removal of histones is required. The protein heterodimer complex FACT consists of two proteins Spt16 and SSRP1 that bind directly to histone dimers and augment the removal of one histone H2A/H2B dimer (Belotserkovskaya et al., 2003). Concomitant with a lower propensity for Cp to accommodate nucleosomes, FACT binding to Cp in Mutu III cells was equivalent to that of Mutu I cells (Figure 17B). Interestingly FACT was detected in large amounts at the W repeat region, suggesting a functional role for FACT during Pol II elongation through Wp. Furthermore FACT was not detected at the LMP gene locus (Figure 17C); suggesting nucleosome remodelling was not required to the same extent as Cp transcription through the W repeats.

### **3.9. NELF protein expression cannot be knocked down using Accell reagent**

Recently NELF was shown to be required for the efficient transcription of the majority of *Drosophila* genes, forming a barrier to nucleosome assembly around the promoter (Gilchrist et al., 2010, Gilchrist et al., 2008). Using siRNA directed against NELF-A, we planned to compare the histone occupancy at Cp in the presence or absence of NELF through ChIP assays. We postulated that NELF could play an important role in the displacement of nucleosomes to recruit TFIID and Pol II to Cp. To elucidate the role of NELF in Cp transcription, we used siRNA targeting NELF-A mRNA for knock down studies. Since the use of Accell siRNA reagents required cells to grow in 0-2% serum, we tested the viability of Mutu III cells over a 96 hr period either in the presence or absence of 2% serum. We found no significant difference in Mutu III growth with or without serum using Accell media (Figure 18A). After a 96 hr incubation with



**Figure 17. FACT is recruited to aid transcription through Wp.** (A) Diagram of the LMP gene locus showing the locations of the amplicons generated by the indicated primer sets (1-8). Numbers indicate the 5' end of the forward primer relative to the starts of the LMP1 or LMP2A mRNA sequences (bent arrows) in the B95-8 annotated EBV sequence (NC\_007605). The RBP-J $\kappa$  sites (grey boxes) and PU.1 site (black box) are shown. The vertical arrow indicates the position of the LMP1 polyadenylation site. Results show the mean  $\pm$  standard deviation of three independent ChIP experiments using two chromatin batches from Mutu I (open bars) and Mutu III cell (black bars) using antibodies targeting the FACT subunit Spt16. (B) Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using Cp specific primers. (C) Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using W repeat primers.



**Figure 18. NELF protein expression cannot be effectively knocked-down using Accell siRNA technology.** (A) Mutu III cells were set up at  $4 \times 10^5$  cells/ml for 24 hrs in RPMI-serum containing media. Cells were either left in RPMI (control) or resuspended in Accell media with (black line) or without 2% serum (purple line) for 96 hrs. Live cell counts were determined at 24 hr intervals. (B) PCR amplification of NELF-A specific transcripts. Mutu III cells were untreated or incubated with either control siRNA or NELF-A siRNA for 96 hrs. NELF-A transcripts were measured relative to the control using two NELF mRNA primers. (C) Western blot probing for NELF-A protein after Mutu III cells incubated for 96 hrs with siRNA. Actin was used as a loading control.

NELF-A siRNA, NELF-A transcripts in Mutu III cells were downregulated by 66% compared to levels detected in cells incubated with the non targeting siRNA control (Figure 18B). Unfortunately, western blot analysis revealed NELF-A protein levels were unchanged (Figure 18C), suggesting the NELF-A protein had a long half life. Cycloheximide experiments carried out by Dr Andrea Gunnell investigated the half life of the NELF-A protein in an EBV positive B cell line IB4. After 50 µg/ml cycloheximide treatment for up to 24 hrs, western blot analysis revealed NELF-A protein had a half-life of over 24 hrs (data not shown). Therefore using transient siRNA methods to knock down NELF-A protein in B cells may not be possible.

### 3.10. Discussion

The transcription of the long polycistronic mRNA is critical for the transcription of the EBNAs to augment EBV immortalisation. Upon initial infection, Wp is activated through cellular factors to produce both EBNA-LP and EBNA 2 (Bell et al., 1998, Kirby et al., 2000, Tierney et al., 2000a). EBNA 2 then activates the Cp, leading to long-range transcription and the full panel of EBNA expression (Woisetschlaeger et al., 1991). Including introns, the average human gene length is 10-15 kb (Strachan and Read, 1999). Therefore through the EBNA 2-dependent recruitment of the specialised CTD kinase pTEFb to Cp, Pol II retention is markedly increased to ensure the entire Cp transcript is synthesised. In addition we detected large amounts of paused Pol II in conjunction with pausing factors DSIF and NELF. Promoter-proximal pausing at Cp may play dual positive roles in promoting transcription. Firstly, high-level pausing of Pol II may facilitate the recruitment of pTEFb to Cp to stimulate elongation. Secondly, the occupancy of Pol II with NELF/DSIF may initially prevent the formation of nucleosomes over Cp and then maintain it.

We also show that Cp transcription is distinct from the regulation of the latent membrane protein promoters. Here only a modest level of Pol II, CTD-phosphorylated serines 2 and 5 and DSIF are detectable in comparison to Cp levels. Due to the vast size difference between Cp and each LMP encoded transcript, the recruitment of specialised elongation factors to LMP genes may not be required. Furthermore, the recruitment of FACT to the W repeat region suggests Pol II is required to safely negotiate through a prolonged region of high nucleosome occupancy (about 25 kb). To test the nucleosomal occupancy of the W repeat region, a core histone H3 or H4 ChIP could be performed. In addition, using the EBV type 1 B95-8 sequence (NC\_007605.1), a Wp nucleosome prediction and Cp comparison was carried out (data not shown). Similar to the LMP gene locus, Wp has a much lower propensity to occlude nucleosomes when compared to Cp.

Interestingly, LMP2 transcription units can significantly vary in length because of the terminal repeat elements. Upon entry into host cells, the EBV genome is initially linear with the terminal repeat region being the site of recombination-directed genome circularisation. These regions can span up to 12 kb, therefore LMP2A transcripts can potentially span up to 25 kb in length

(Brown et al., 1988). However, this is still roughly 5 times smaller in size when compared to Cp transcripts and possibly justifies why Cp has specialised elongation features that promote long-range transcription.

Cp promoter-proximal pausing of Pol II appears to be driven by the presence of DNA sequences upstream of Cp that is less favourable for nucleosome assembly. These sequences encompass the TATA box, to potentially allow increased access of TBP, resulting in the high-level recruitment of Pol II in association with the pausing factors NELF and DSIF. Once Pol II stalling has been established at Cp, a more extensive region around the promoter is then maintained in a nucleosome-depleted state. Initially NELF was identified as a negative factor that promotes Pol II pausing at inducible *Drosophila* genes e.g. *Hsp70* (Gilchrist et al., 2008, Lee et al., 2008). Interestingly, studies in both *Drosophila* and humans have shown NELF to act as both a positive and negative regulator of genes (Narita et al., 2007, Gilchrist et al., 2008). Further research has shown NELF enhances nucleosome occlusion at promoter proximal regions to facilitate transcription (Gilchrist et al., 2008). Since we detected high-level accumulation of paused Pol II and NELF concomitant with low core histone H3 occupancy, we postulated that knock down of NELF-A would increase histone levels and decrease Cp transcription. However, due to the long half life of the NELF-A protein in Mutu III cells we were unable to deduce a role for NELF at Cp. Future experiments could target other NELF components for knock down such as NELF-B or NELF-C (Sun et al., 2008). Moreover, using a lentiviral expression system, producing shRNA to target NELF components could be a highly effective strategy.

Interestingly, DSIF component Spt5 was detected at both C promoter proximal and distal regions eluding to a possible positive role in Cp transcription. Phosphorylation of Spt5 by pTEFb is known to switch DSIF into a positive-acting elongation factor, stimulating Pol II processivity and preventing premature termination (Wada et al., 1998a). Spt5 has been shown to promote transcriptional activation by Gal4-VP16 and is recruited to the HIV-1 LTR to co-operate in the stimulation of transcriptional elongation by HIV-1 Tat (Zhu et al., 2007, Bourgeois et al., 2002). More recently, *Drosophila* studies of the *Hsp70* gene locus have revealed that both NELF-E and Spt5 can contact nascent RNA in a transcript-length dependent manner and that NELF may be recruited to Pol II complexes through DSIF (Missra and Gilmour, 2010). Also, siRNA studies to

determine whether DSIF recruits NELF to Cp and whether nucleosome occlusion across Cp is DSIF-dependent could be very important in understanding promoter switching during EBV latent infection. It would also be interesting to determine whether over-expression of both NELF and DSIF components in EBV latency I cell lines could activate Cp through nucleosome occlusion, switching to a latency III regulatory programme.

Both CDK7 and CDK8 have previously been shown to phosphorylate the CTD of Pol II and regulate transcriptional initiation and elongation (Akoulitchev et al., 1995, Komarnitsky et al., 2000, Marshall et al., 1996, Rickert et al., 1996). CDK7 is part of the basal transcription machinery complex TFIIH and requires Cyclin H for kinase activity (Fisher and Morgan, 1994). Conversely CDK8 and partner Cyclin C can interact with mediator complexes (Furumoto et al., 2007). In previous ChIP studies both CDK7 and CDK8 have been shown to bind at promoters and stimulate transcription through Pol II CTD phosphorylation (Gomes et al., 2006, Meyer et al., 2008). In this study we detected high level Pol II serine 5 CTD phosphorylation at C proximal and distal regions and expected to detect CDK7 and possibly CDK8 at Cp. However both CDK7 and CDK8 did not IP in Mutu cells under varying ChIP conditions. The likelihood that CDK7 is the kinase responsible for Cp CTD serine 5 phosphorylation is much greater than that of CDK8 because of the absence and negative role of CDK8 in most mediator complexes (Taatjes, 2010). We suspect using different antibodies or by targeting other proteins commonly found in TFIIH (CDK7) or mediator (CDK8) complexes such as their Cyclin partners may prove more rewarding. Alternatively, using drugs designed to inhibit either CDK7 (BS-181) or CDK8 (H7) kinase activity could help to elucidate their potential roles at Cp (Ali et al., 2009, Belakavadi and Fondell, 2010).

EBV strains 1 and 2 can both transform B cells *in vitro*, however type 1 strains can establish transformants much more efficiently than type 2 (Rickinson et al., 1987). This has been attributed to the low EBNA 2 gene homology between the two strains (Addinger et al., 1985, Dambaugh et al., 1984). Recently, type I EBNA 2 was shown to induce LMP1 and various cellular genes quicker and in larger quantities when compared to type 2 EBNA 2 (Lucchesi et al., 2008). However, the results described here demonstrate that high-level pTEFb recruitment is driven by Pol II stalling, initiated by the Cp DNA sequence promoting reduced nucleosome occupancy, rather than variations in the EBNA 2 primary sequence. To investigate whether type



2 Cp sequences also possessed this property, we performed nucleosome occupancy predictions using the sequences of the type 2 viral strain AG876. Our results suggested that the nucleosome occupancy at the type 2 C, LMP and W promoters was predicted to be virtually indistinguishable (data not shown) from the type 1 B95-8 strain previously examined. This is perhaps not surprising given the high sequence homology surrounding the latent promoter sequences in both strains. Therefore type 2 viruses retain the same ability to occlude nucleosomes as type 1 strains and it is unlikely that pTEFb recruitment would significantly contribute to the transforming potential of type 2 viruses.

In summary, we demonstrate that Cp-dependent polymerase stalling may play a role in augmenting immortalisation of B cells by the tumour virus EBV. High-level recruitment of Pol II and associated pausing factors NELF and DSIF to the viral C promoter maintains nucleosome depleted regions to facilitate substantial pTEFb recruitment to overcome pausing. This provides high levels of pTEFb to promote the distal serine 2 CTD phosphorylation required for production of the long viral transcript encoding key EBV immortalizing genes.

#### 4. Investigating the Cp-specific recruitment of the Pol II CTD kinase pTEFb

Our previous results demonstrated high-level recruitment of both pTEFb components CDK9 and Cyclin T1 to Cp, with DRB-mediated CDK9 inhibition resulting in the decreased retention of Pol II and ser 2 CTD phosphorylation at Cp distal regions. Our next investigation focussed on the requirement for pTEFb for EBV transcription *in vivo* and the mechanism of recruitment to Cp.

In the nucleus pTEFb exists in a state of equilibrium, with approximately half found inactive and bound in a high molecular weight complex to HEXIM and 7SK snRNA (Yang et al., 2001, Nguyen et al., 2001). Cellular stresses such as heat shock, UV exposure, actinomycin D treatment and DRB/flavopiridol-mediated CDK9 inhibition disrupt this equilibrium, allowing the dissociation of pTEFb from its inactive complex (Biglione et al., 2007, Nguyen et al., 2001, Yang et al., 2001). The equilibrium shifts through the activation of signalling pathways leading to post-translational modifications such as the phosphorylation and acetylation of CDK9 (Ammosova et al., 2011a, Zhou et al., 2009, Wang et al., 2008a, Chen et al., 2008, Ammosova et al., 2005). Once released pTEFb is specifically recruited to promoters through activators to facilitate efficient transcription of genes through phosphorylation of the Pol II CTD and transcription factors involved in promoter-proximal pausing. CDK9 also mediates epigenetic histone modifications either directly or indirectly through phosphorylating the CTD (Chao and Price, 2001, Marshall and Price, 1995, Kim and Sharp, 2001, Fujinaga et al., 1998, Wada et al., 1998b, O'brien et al., 2010, O'brien et al., 2011, Pirngruber et al., 2009).

Several different proteins such as Brd4 and the HIV-1 tat protein are capable of binding to and recruiting pTEFb to both cellular and viral promoters to stimulate transcriptional elongation (Zhou et al., 2006, Qi et al., 2011, Yang et al., 2005, Jang et al., 2005, Zhu et al., 1997, Mancebo et al., 1997). Brd4 is a ubiquitously expressed cellular protein that contains two bromodomains capable of interacting with di-acetylated lysine residues on histone H3 and H4 tails (AcH3 and AcH4) and pTEFb simultaneously, providing a link between epigenetic regulation and transcriptional elongation (Dey et al., 2003, French et al., 2001, Liu et al., 2008,

Vollmuth et al., 2009, Jang et al., 2005, Yang et al., 2005). Additionally Brd4 has been shown to facilitate transcriptional regulation of several viral genomes such as HPV, KSHV, HTLV-1 and HIV-1 (Bisgrove et al., 2007, Cho et al., 2007, You et al., 2006, Wu et al., 2006).

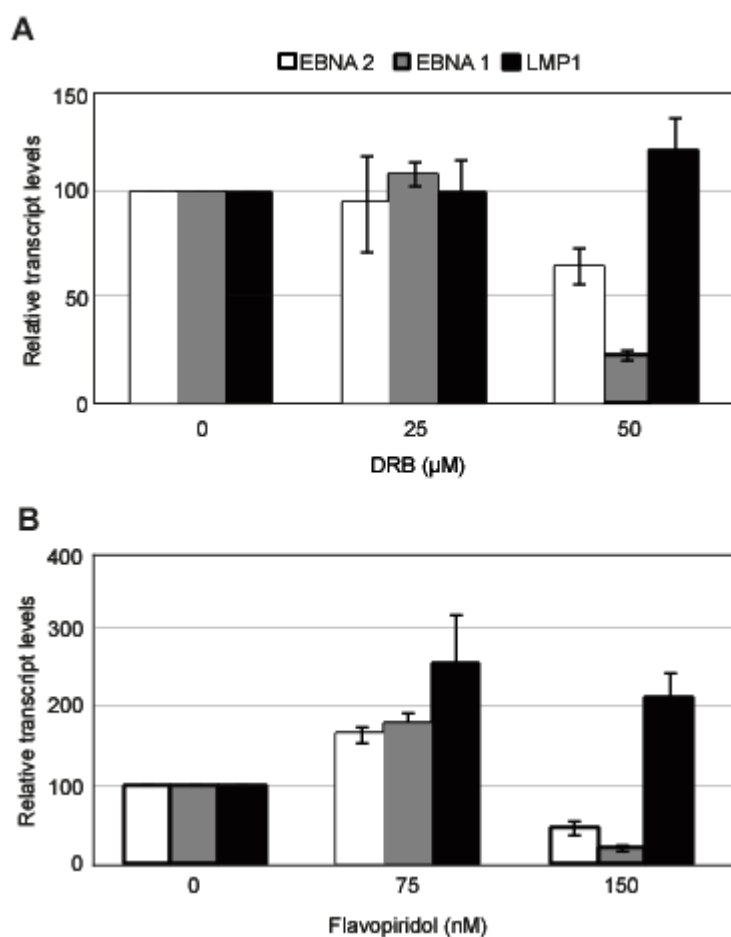
#### **4.1. Cp transcription is more sensitive to CDK9 inhibitors DRB and Flavopiridol**

Flavopiridol (or Alvocidib) was originally derived from an indigenous Indian plant, it has undergone clinical trials against numerous cancers including lymphocytic leukemias because of its pro-apoptotic nature (Stadler et al., 2000, Shapiro et al., 1999, Senderowicz et al., 1998, Byrd et al., 1998). Acting as an ATP analogue to CDK9 and other cyclin dependent kinases (CDKs), it induces structural changes preventing the binding of ATP to the active site, inhibiting kinase activity (De Azevedo et al., 2002, Baumli et al., 2008). Our laboratory has previously found that Flavopiridol decreases EBNA 2 dependent Cp-transcription with an  $IC_{50}$  of 83 nM in luciferase reporter assays (unpublished results).

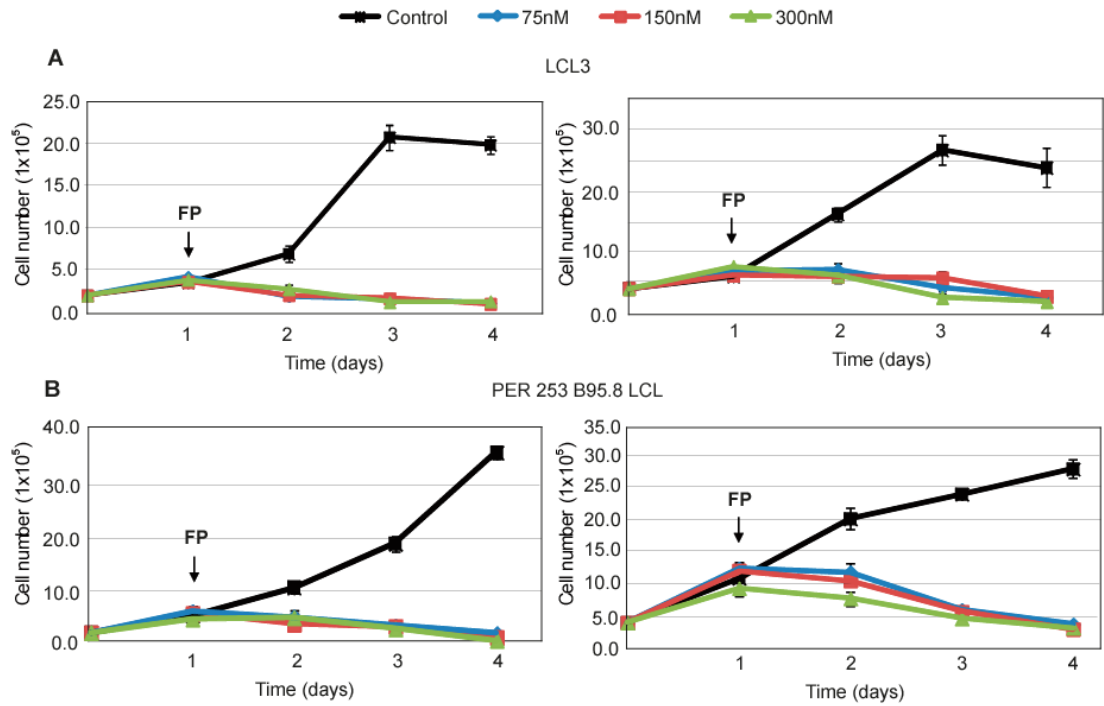
Since the pTEFb inhibitor DRB inhibited Pol II retention and serine 2 CTD phosphorylation (Figure 9), we investigated the effects of the pTEFb inhibitors DRB and Flavopiridol on Cp and LMP1p transcription in Mutu III cells. Consistent with the selective high-level recruitment of pTEFb to Cp *in vivo*, we found that the pTEFb inhibitors DRB and Flavopiridol were both able to inhibit Cp transcription at concentrations at which LMP1 transcription was unaffected (Figure 19). After a 24 hr incubation with 50  $\mu$ M DRB, Mutu III EBNA 1 Cp transcripts were inhibited by approximately 80% compared to the DMSO control (Figure 19A). In contrast the level of LMP1 transcripts were unaffected. Similarly, 150 nM Flavopiridol treatment inhibited Cp transcription by 80% (Figure 19B).

#### **4.2. LCL growth is severely inhibited by Flavopiridol treatment**

Since Flavopiridol could strongly inhibit Cp transcription and EBNA 2 transcript levels (Figure 19B), we tested whether Flavopiridol treatment could prevent LCL growth. Both LCL3 and PER 253 B95.8 LCL were treated with 75 nM, 150 nM or 300 nM Flavopiridol over a period of 4 days.



**Figure 19. pTEFb inhibitors selectively reduce Cp transcription.** Mutu III cells were treated with the indicated concentrations of DRB (A) or Flavopiridol (B) for 24 hrs and transcript levels determined using the specific Q-PCR primers indicated and actin as an endogenous control. Normalised cDNA levels are expressed relative to 24 hr control samples. Results show the mean  $\pm$  standard deviation of two independent experiments.



**Figure 20. Flavopiridol inhibits LCL growth.** (A) LCL3 and (B) PER 253 B95.8 LCL were set up at either  $2 \times 10^5$  (left panels) or  $4 \times 10^5$  cells/ml (right panels) 24 hrs prior to treatment. Cells were either untreated (control) or incubated with 75, 150 or 300 nM of the CDK9 inhibitor Flavopiridol (FP) after 24 hrs for a further 96 hrs. The arrows indicate the addition of Flavopiridol. Live cell counts were determined at 24 hr intervals. Results show the mean  $\pm$  standard deviation of eight individual cell counts.

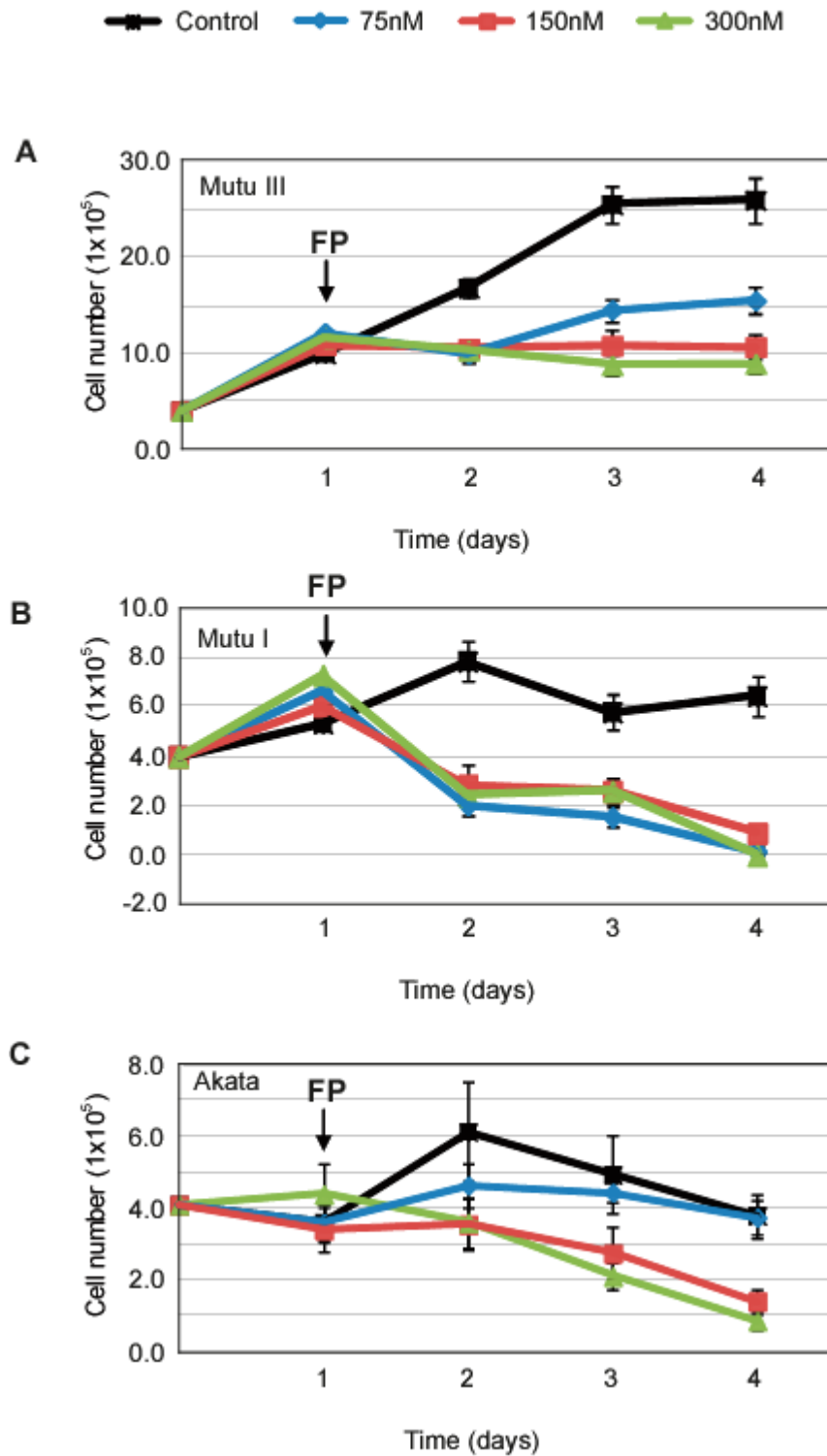
Figures 20A and B clearly demonstrate the growth inhibiting effects of Flavopiridol on both LCLs at all concentrations.

We next compared the effects of Flavopiridol on Mutu I and Mutu III cells. Mutu III cells were less sensitive to Flavopiridol treatment when compared to both LCLs. Moreover, the 75 nM Flavopiridol treatment was not sufficient enough to prevent some Mutu III continued cell growth (Figure 21A). However, growth appeared arrested at the higher 150 nM and 300 nM Flavopiridol concentrations.

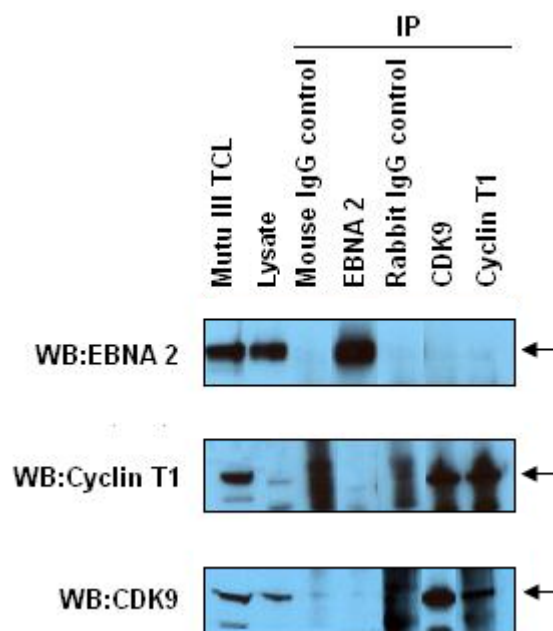
We observed that Mutu I cells were much more sensitive to Flavopiridol treatment than Mutu III cells (Figure 21B). Flavopiridol experiments using the Akata cell line, another Group I BL, produced similar results to Mutu I (Figure 21C). This aligns with previous data showing that anti-apoptotic effects are induced in the presence of type III viral proteins such as EBNA 2 and EBNA 3C (Lee et al., 2002, Anderton et al., 2008, Kelly et al., 2009, Gregory et al., 1991). In summary, Flavopiridol can inhibit both LCL and type I BL cell growth, with latency I BL cells showing characteristically increased sensitivity to treatment with this cytotoxic drug. Unfortunately, it is not possible to determine from more experiments whether these results are due to the loss of EBNA 2 production.

### **4.3. EBNA 2 does not associate with pTEFb in Mutu III cells**

The associations of pTEFb with Cp in the presence of EBNA 2 led us to determine whether EBNA 2 directly recruited pTEFb. Previous work in our laboratory involving the over-expression of EBNA 2 and pTEFb in EBV negative B cells did not detect any interactions between CDK9 and Cyclin T1 with EBNA 2. To further investigate potential interactions, we immunoprecipitated endogenous EBNA 2, CDK9 and Cyclin T1 from Mutu III cells and examined their potential association by western blotting for co-precipitated proteins. Our results showed efficient precipitation of EBNA 2 but failed to detect any interactions with pTEFb (Figure 22, top panel). However co-immunoprecipitations can fail to detect potential protein interactions if antibody access to epitopes is blocked by the interaction. We therefore also immunoprecipitated CDK9 and Cyclin T1 proteins and determined whether EBNA 2 was co-precipitated. Although both



**Figure 21. Flavopiridol inhibits Group I and III Burkitt's Lymphoma cells.** (A) Mutu III (B) Mutu I and (C) Akata cells were set up at  $4 \times 10^5$  cells/ml 24 hrs prior to treatment. Cells were either untreated (control) or incubated with 75, 150 or 300 nM of the CDK9 inhibitor Flavopiridol (FP) after 24 hrs for a further 96 hrs. The arrows indicate the addition of Flavopiridol. Live cell counts were determined at 24 hr intervals. Results show the mean  $\pm$  standard deviation of eight individual cell counts.



**Figure 22. EBNA 2 does not interact directly with the pTEFb subunits CDK9 and Cyclin T1 in Mutu III cells.** EBNA 2 and pTEFb subunits were immunoprecipitated (IP) using either protein A or protein G sepharose beads from Mutu III cells. Western blots (WB) probed IPs and total cell lysates (TCL) for EBNA 2 (top panel), Cyclin T1 (middle panel) and CDK9 (bottom panel). The rabbit IgG IP served as controls for both pTEFb rabbit antibodies whilst the mouse IgG IP served as a negative control for the EBNA 2 IP. The arrows on the right indicate where the protein should be and distinguishes between real and background signals.

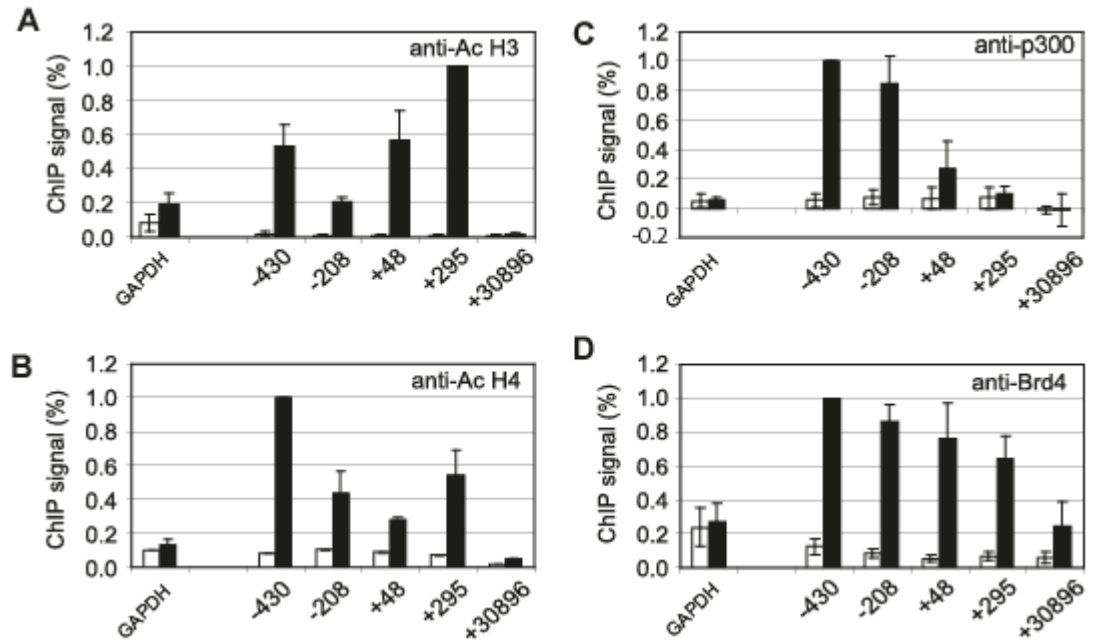


CDK9 and Cyclin T1 proteins interacted with each other as expected, neither interacted with EBNA 2 (Figure 22 middle and bottom panels), confirming the previous findings in transient over-expression assays.

#### **4.4. High-level histone acetylation, p300 and Brd4 are present at Cp in the presence of EBNA 2**

The inability to detect an interaction between EBNA 2 and pTEFb led us to investigate a potential role for Brd4 in pTEFb recruitment. Initial ChIP experiments confirmed high-level di-acetylated lysine residues on histones H3 (K9 and K14) and H4 (K5 and K12) at the C promoter in Mutu III cells (Figures 23A and B), consistent with active transcription and previous observations (Fejer et al., 2008, Day et al., 2007). Interestingly both di-acetylated histones gave different ChIP profiles, with the AcH3 signal peaking between +295 and +406 (Figure 23A) whilst the AcH4 signal peaked between -430 and -337 (Figure 23B). Further ChIP assays examined the association of the histone acetyltransferase p300 with Cp, p300 is known to interact with EBNA 2 and acetylate both histones H3 and H4 (Wang et al., 2000, Schiltz et al., 1999). Consistent with directed recruitment via EBNA 2, we detected large amounts of p300 around the Cp RBPJk site (-430 to -337 primer set) and peak of EBNA 2 binding (Figure 23C). ChIP assays next investigated Brd4 association with Cp. Significantly, we detected large quantities of Brd4 at all Cp proximal regions (Figure 23D). These data may therefore implicate Brd4 in pTEFb recruitment to Cp either through a direct EBNA 2-Brd4 interaction or indirectly through p300 recruitment and acetylation of histones.

Recently, Brd4 was shown to bind to the Family Repeat element (FR) region of the EBV genome and augment transcription of EBNA 1-dependent genes (Lin et al., 2008). The FR element is part of the latent origin of replication (OriP) and contains multiple copies of an 18 base pair palindromic EBNA 1 binding sequence important for host chromosome tethering (Reisman et al., 1985, Rawlins et al., 1985, Reisman and Sugden, 1986). Additionally OriP functions as a distal Cp enhancer (Nilsson et al., 2001, Puglielli et al., 1996). We therefore investigated the possibility that EBNA 1 may recruit pTEFb to OriP via Brd4 and contribute to the level of pTEFb at Cp through DNA looping effects. To investigate this possibility, we



**Figure 23. Brd4 is recruited to Cp through acetylated histones.** Percentage input signals, after subtraction of no antibody controls are expressed for comparison purposes relative to the highest signal obtained. ChIP was performed in Mutu I cells (open bars) and Mutu III cells (black bars) using anti-acetyl Histone H3 (n=3, c=2) (A), anti-acetyl Histone H4 (n=5, c=2) (B), anti-p300 (n=3, c=2) (C) and anti-Brd4 antibodies (n=3, c=2) (D). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.

examined Brd4 association with OriP at the FR element. ChIP assays detected some Brd4 binding to OriP in Mutu I and Mutu III cells, equivalent to that detected in the GAPDH gene (Figure 24A). However, the level of Brd4 detected was much lower than that at Cp and it did not result in any significant recruitment of pTEFb (figure 24B and C). These data therefore indicate that OriP does not play a role in high-level recruitment of pTEFb and Brd4 to Cp.

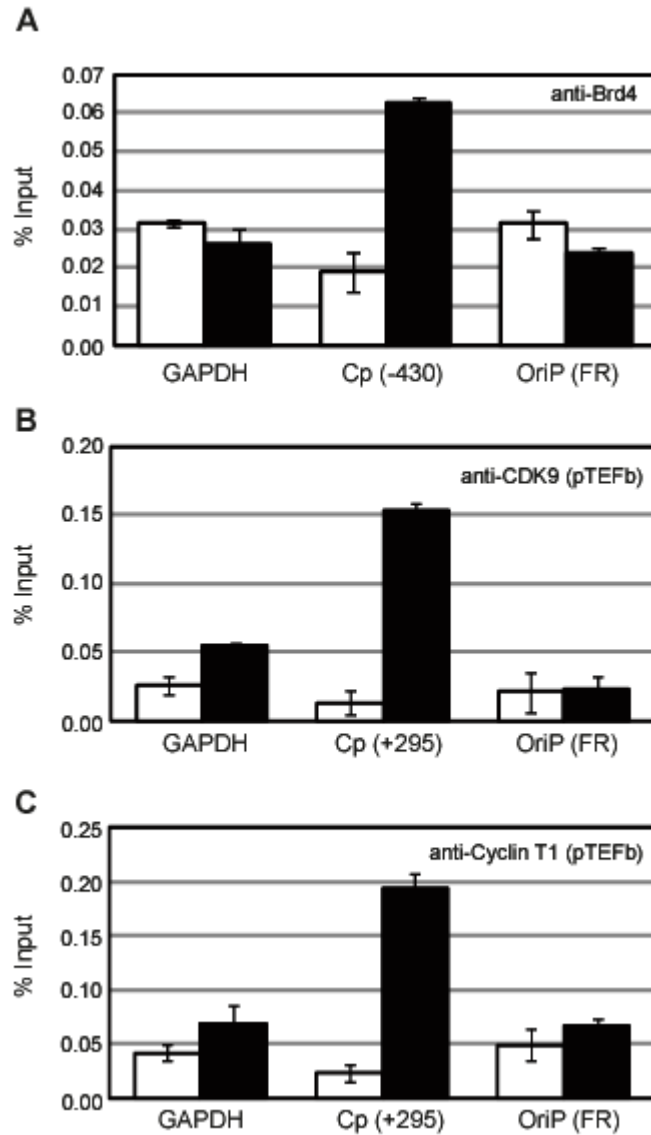
#### **4.5. Brd4 is also recruited to the LMP1 and LMP2A promoters**

Further experiments examined histone acetylation and Brd4 recruitment at the other latent promoters LMP1 and LMP2A. In agreement with past experiments (Gerle et al., 2007, Alazard et al., 2003), we found that Ach3, Ach4 and p300 levels were increased in Mutu III cells (Figures 25A-C). Our previous ChIP assays did not detect significant levels of pTEFb at the LMP gene locus (Figures 12B and C). Despite the lack of detectable pTEFb, ChIP assays identified Brd4 protein at both LMP promoters (figure 25D).

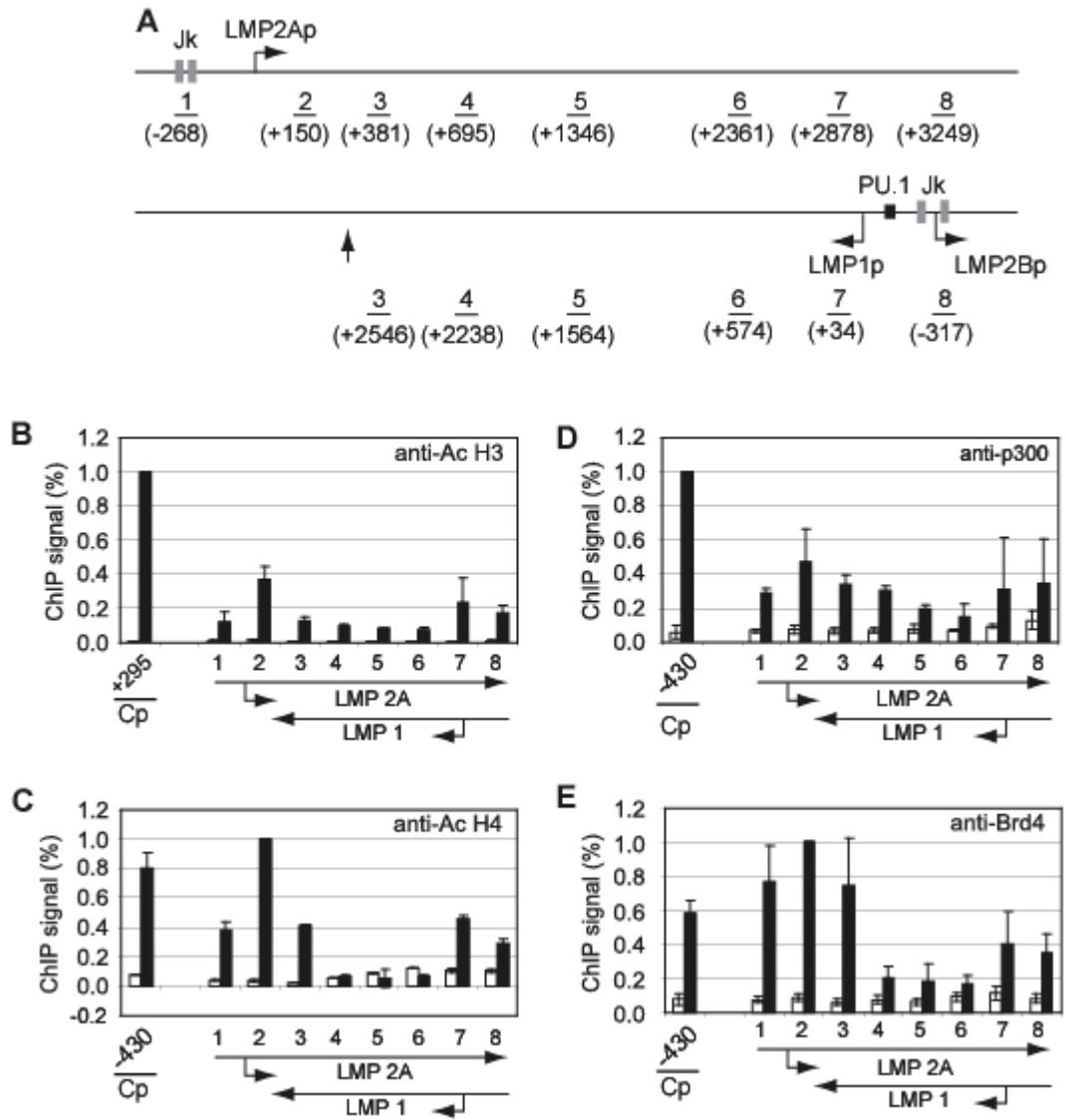
To determine whether Brd4 was recruited to all EBV latent promoters at an equivalent level in another cell background, further ChIP experiments were carried out in an LCL. We found that both Ach4 and Brd4 ChIP profiles were very similar to those detected in Mutu III cells (Figures 26B and C). Interestingly the recruitment of Brd4 to the LMP gene locus does not coincide with any detectable pTEFb recruitment, indicating pTEFb recruitment cannot be solely mediated by Brd4 and Brd4 may have another role in LMP transcription.

#### **4.6. Brd4 does not interact with EBNA 2 in Mutu III cells**

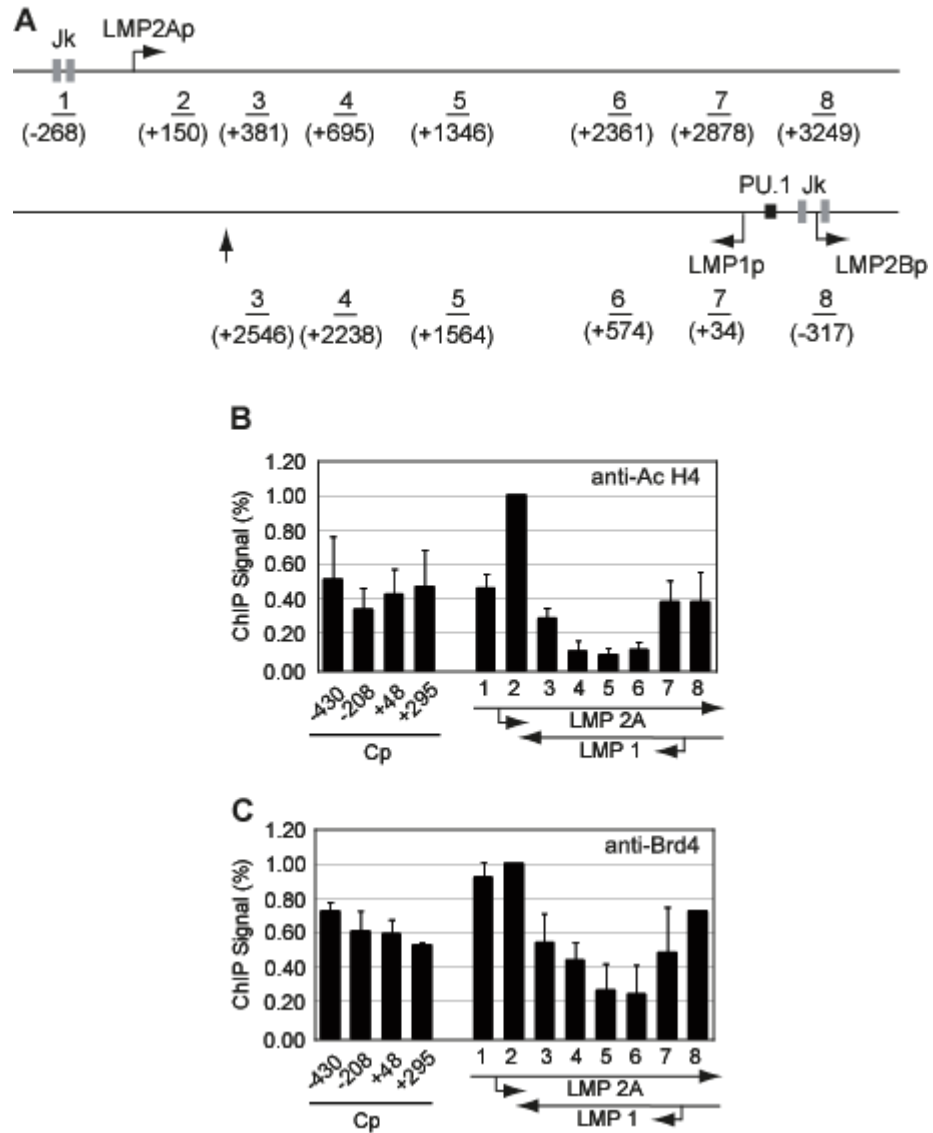
Since Brd4 can be recruited to promoters by transcriptional activators and both EBNA 2 and Brd4 displayed similar binding profiles across Cp, we investigated whether EBNA 2 could indirectly recruit pTEFb to Cp through interactions with Brd4. Immunoprecipitations of Brd4, EBNA 2, CDK9 and Cyclin T1 were carried out in Mutu III cells using western blotting to probe for interactions between them. As expected, immunoprecipitations carried out using CDK9 and Cyclin T1 antibodies successfully precipitated Brd4 (Figure 27, top panel). However, EBNA 2 was not detected as a co-precipitated protein. EBNA 2 immunoprecipitations also failed to detect co-precipitation of Brd4 (Figure 27, bottom panel). CDK9 and Cyclin T1 were not co-



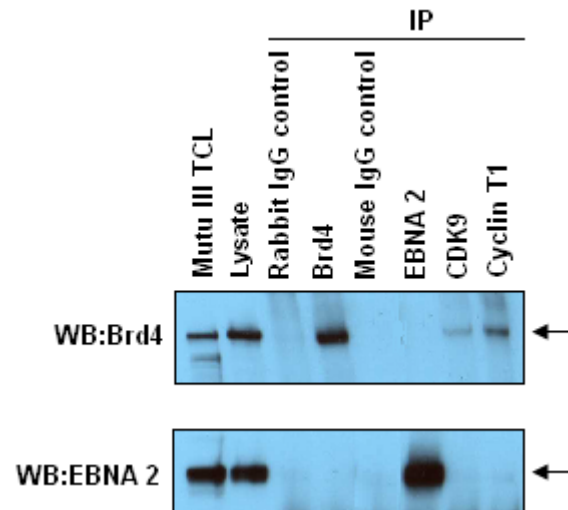
**Figure 24. pTEFb is not recruited to OriP at high levels.** Results show the mean percentage input signal (after subtraction of the no antibody control signal)  $\pm$  standard deviation of two independent experiments using Mutu I (open bars) and Mutu III cell chromatin (black bars). ChIP using (A) anti-Brd4 ( $n=2$ ,  $c=1$ ), (B) anti-CDK9 ( $n=2$ ,  $c=1$ ) and (C) anti-cyclin T1 antibodies ( $n=2$ ,  $c=1$ ). Cp analysis was carried out with the primer set that gave the highest signal for each transcription factor. OriP primers are adjacent to the EBNA 1 binding element (family of repeats, FR) (Appendix D). Where  $n$  denotes the number of experimental repeats and  $c$  denotes the number of chromatin batches.



**Figure 25. Brd4 is recruited to LMP1p in Mutu III cells.** (A) Diagram of the LMP gene locus showing the locations of the amplicons generated by the indicated primer sets (1-8). Numbers indicate the 5' end of the forward primer relative to the starts of the LMP1 or LMP2A mRNA sequences (bent arrows) in the B95-8 annotated EBV sequence (NC\_007605). The RBP-J $\kappa$  sites (grey boxes) and PU.1 site (black box) are shown. The vertical arrow indicates the position of the LMP1 polyadenylation site. Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using Cp-specific primers. ChIP was performed with Mutu I (open bars) and Mutu III cell chromatin (black bars) using anti-AcH3 antibodies (n=4, c=3) (B), anti-AcH4 antibodies (n=3, c=2) (C), anti-P300 antibodies (n=3, c=2) (D) and anti-Brd4 antibodies (n=3, c=2) (E). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.



**Figure 26. Brd4 is recruited to LMP1p in PER 253 cells.** (A) Diagram of the LMP gene locus showing the locations of the amplicons generated by the indicated primer sets (1-8). Numbers indicate the 5' end of the forward primer relative to the starts of the LMP1 or LMP2A mRNA sequences (bent arrows) in the B95-8 annotated EBV sequence (NC\_007605). The RBP-J $\kappa$  sites (grey boxes) and PU.1 site (black box) are shown. The vertical arrow indicates the position of the LMP1 polyadenylation site. Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained. ChIP with Mutu I (open bars) and Mutu III cell chromatin (black bars) using anti-AcH4 antibodies (n=4, c=2) (B) and anti-Brd4 antibodies (n=3, c=2) (C). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.



**Figure 27. Brd4 does not directly interact with EBNA 2 in Mutu III cells.** Brd4, EBNA2 and pTEFb subunits were Immunoprecipitated (IP) using either protein A or protein G sepharose beads from Mutu III cells. Western blots (WB) probed each IP and total cell lysates (TCL) for interactions with either Brd4 (top panel) or EBNA 2 (bottom panel). The rabbit IgG IP served as negative controls for both Brd4 and pTEFb subunits and the mouse IgG IP served as a control for the EBNA 2 IP. The arrows on the right indicate where the protein should be and distinguishes between real and background signals.

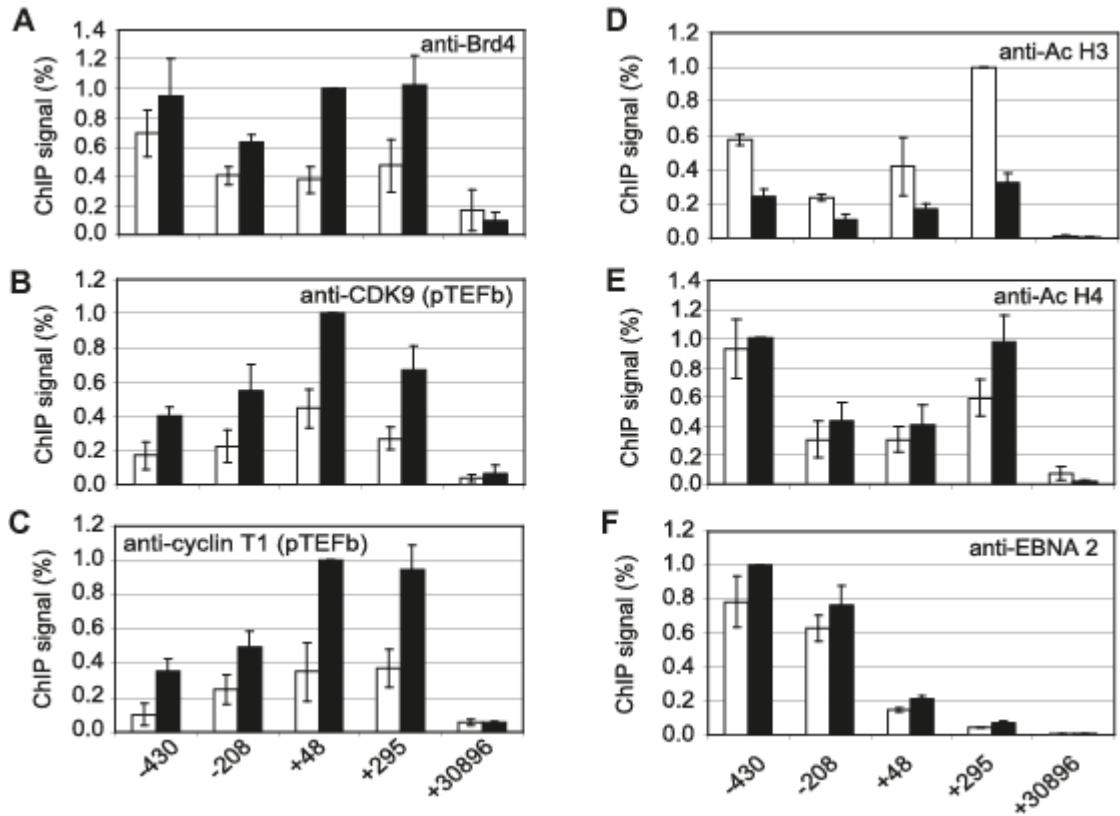
precipitated as previously observed (Figure 22). These data indicate that any Brd4-dependent recruitment of pTEFb is not simply mediated via interactions with EBNA 2. These data are consistent with our observations that Brd4 is associated with all EBNA 2-activated latency promoters (Cp, LMP1p and LMP2Ap) but pTEFb is specifically recruited to Cp presumably via a mechanism that does not involve simple recruitment of either Brd4 or pTEFb by EBNA 2.

#### **4.7. Pol II stalling stabilizes pTEFb recruitment to Cp via Brd4**

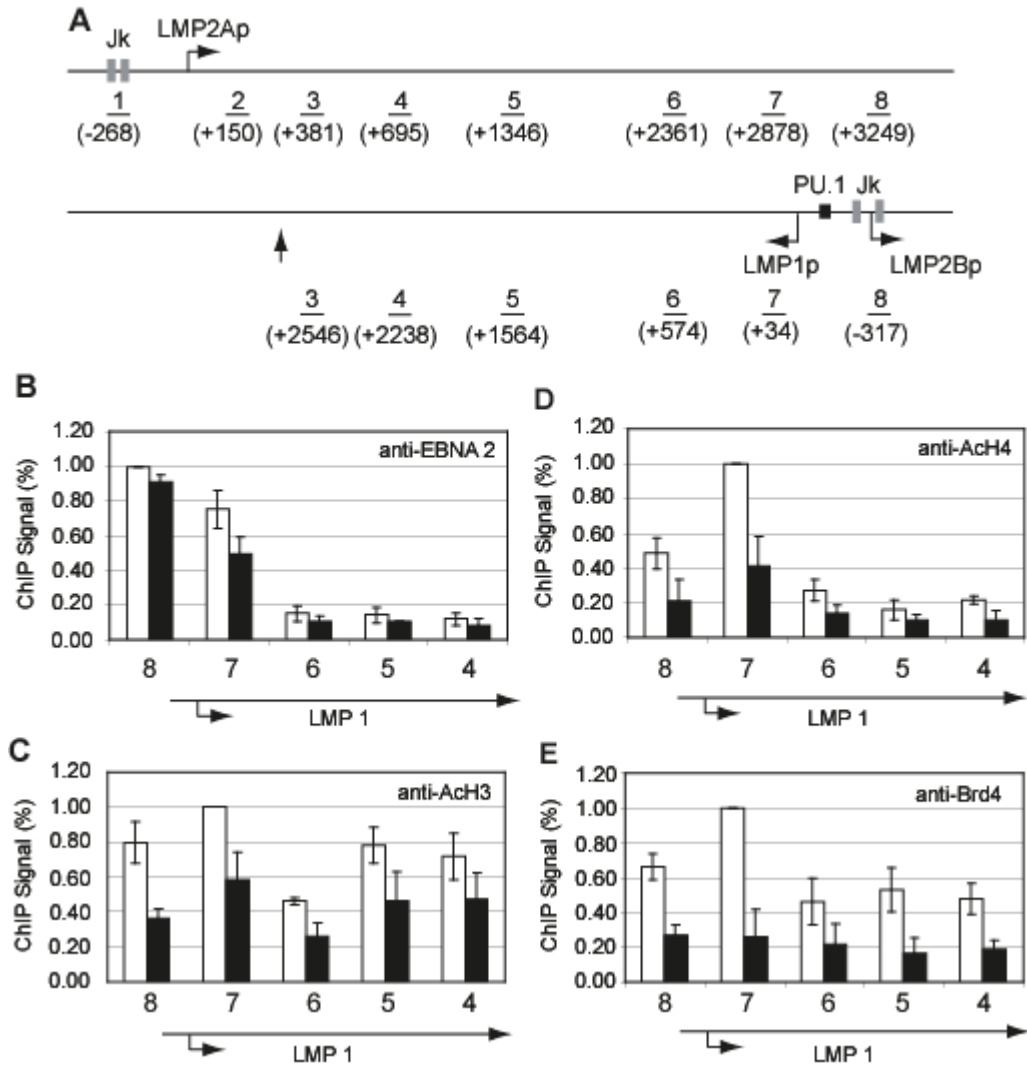
Cellular stresses such as pTEFb inhibition cause the dissociation of pTEFb from its inactive complex, allowing the binding of Brd4 and recruitment to active genes (Yang et al., 2005, Jang et al., 2005). To elucidate a role for Brd4 at EBNA 2-activated latency promoters, we performed ChIP assays in the presence of DRB. We observed that in the presence of DRB, a 2-fold increase in the levels of pTEFb correlated with Brd4 at Cp (Figures 28A, B and C). Ach4 was increased by DRB treatment at Cp, perhaps as a result of the protection from deacetylation provided by the preferential binding of Brd4 to acetylated Histone H4 residues (Dey et al., 2003). Our data does seem to indicate that the pattern of Brd4 binding more closely resembles the profile of histone H4 rather than histone H3 acetylation (Figures 28D and E). Furthermore in agreement with our previous findings, the increased pTEFb/Brd4 recruitment to Cp was independent of EBNA 2 levels (Figure 28F).

In sharp contrast, DRB treatment led to loss of Brd4 from the LMP1 promoter and decreases in Histone H3 and H4 acetylation (Figure 29). Since the key difference between the C and LMP1 promoters is the presence of high levels of stalled Pol II at Cp, these results suggest that pTEFb is efficiently recruited to Cp via Brd4 as a result of stable interactions between pTEFb and the large numbers of stalled polymerases present at the promoter. Thus at LMP1p, in the absence of an accumulation of Pol II molecules, pTEFb complexes brought in by Brd4 have little Pol II with which to stably associate.





**Figure 28. Increased recruitment of Brd4 to Cp in response to DRB treatment.** Percentage input signals, after subtraction of no antibody controls are expressed for comparison purposes relative to the highest signal obtained. ChIP with Mutu III cells in the absence (open bars) or presence of 500  $\mu$ M DRB (black bars) for 2 hrs using anti-Brd4 (n=3, c=2) (A) anti-CDK9 (n=3, c=2) (B) anti-Cyclin T1 (n=3, c=2) (C) anti-acetyl Histone H3 (n=3, c=2) (D) anti-acetyl Histone H4 (n=5, c=2) (E) and anti-EBNA 2 antibodies (n=3, c=2) (F). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.



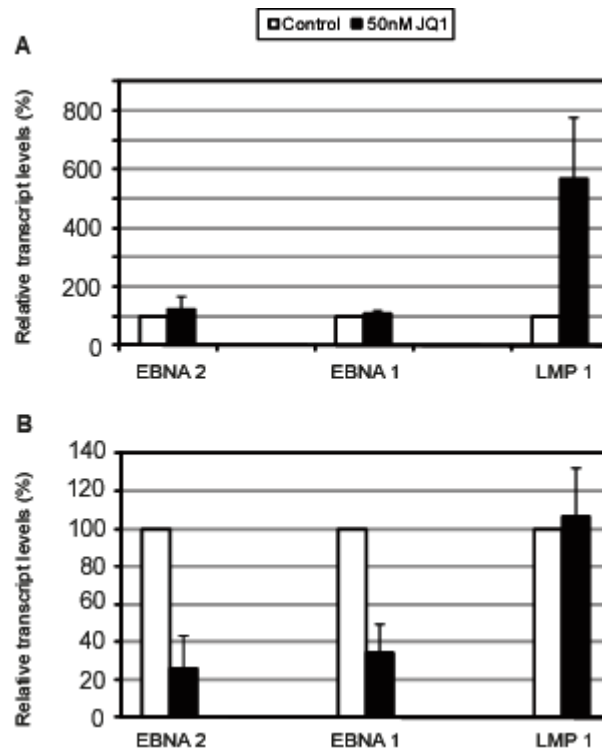
**Figure 29. DRB treatment decreases histone acetylation and Brd4 recruitment to the LMP1 promoter.** (A) Diagram of the LMP gene locus showing the locations of the amplicons generated by the indicated primer sets (1-8). Numbers indicate the 5' end of the forward primer relative to the starts of the LMP1 or LMP2A mRNA sequences (bent arrows) in the B95-8 annotated EBV sequence (NC\_007605). The RBP-J $\kappa$  sites (grey boxes) and PU.1 site (black box) are shown. The vertical arrow indicates the position of the LMP1 polyadenylation site. Percentage input signals, after subtraction of no antibody controls, are expressed relative to the highest signal obtained using LMPp-specific primers. ChIP carried out in Mutu III cells minus (open bars) or plus 500  $\mu$ M DRB (black bars) using anti-EBNA 2 (n=3, c=2) (B), anti-acetyl histone H3 (n=5, c=2) (C), anti-acetyl histone H4 (n=3, c=2) (D) and anti-Brd4 antibodies (n=3, c=2) (E). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.

#### 4.8. Brd4 inhibition reduces pTEFb recruitment

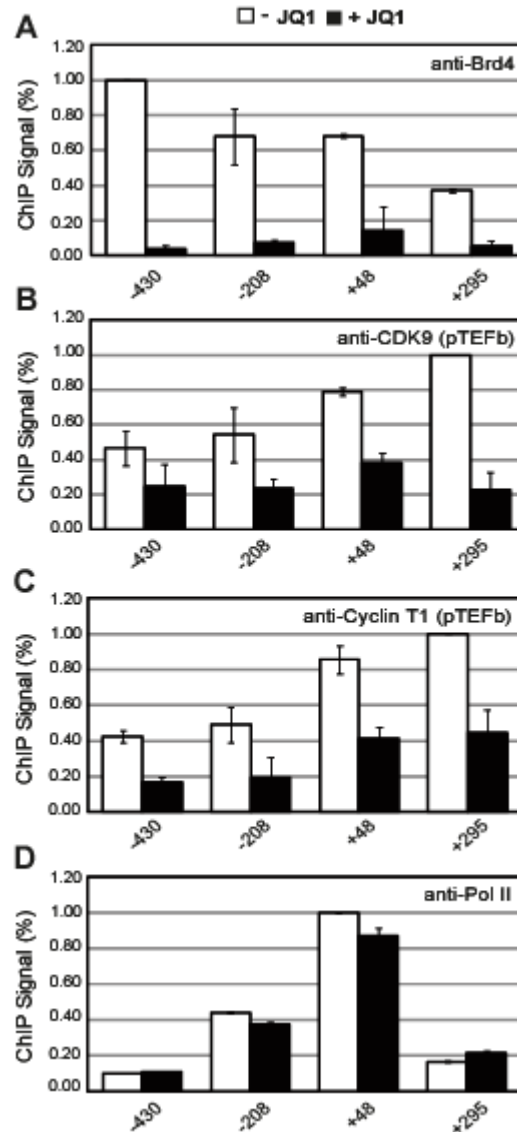
Recently, a small molecule Brd4 inhibitor, JQ1, has been shown to function as a competitive inhibitor to prevent both Brd4 bromodomains binding to acetylated histones (Filippakopoulos et al., 2010). To determine whether Brd4 is required for EBV latent transcription, Mutu III cells were incubated with 50 nM JQ1 for either 24 or 48 hrs and both EBNA 1 and EBNA 2 Cp, and LMP1p derived transcripts were quantified (Figure 30). After 24 hrs, JQ1 had no significant effect on either EBNA 1 or EBNA 2 transcripts (black bars). Surprisingly, JQ1 treatment increased LMP1p transcripts by nearly 6 fold (Figure 30A). Strikingly, treatment with 50nM JQ1 for 48 hrs reduced levels of Cp-initiated EBNA 2 and EBNA 1 transcripts by 74% and 65% respectively, but had no effect on LMP1 transcript levels (Figure 30B). These data indicate that Cp and not LMP1p is dependent on Brd4 for its transcription

Previously we detected a direct correlation between Brd4 and pTEFb recruitment to Cp in response to DRB (Figure 28A-C). To determine whether pTEFb recruitment to Cp was Brd4 dependent we treated Mutu III cells with 50 nM JQ1 for 48 hrs and prepared chromatin for ChIP assays. Our results demonstrated that JQ1 inhibited Brd4 binding to Cp by 96% at -430 to -337 and 79% at +48 to +167 (Figure 31A). We also detected a corresponding decrease in both CDK9 and Cyclin T1 binding in the presence of JQ1 (Figures 31B and C). Control experiments demonstrated that there were no significant effects on pre-initiation complex assembly as a result of JQ1 treatment since Pol II recruitment remained largely unchanged (Figure 31D). These data demonstrate that Brd4 is required for pTEFb recruitment to Cp.

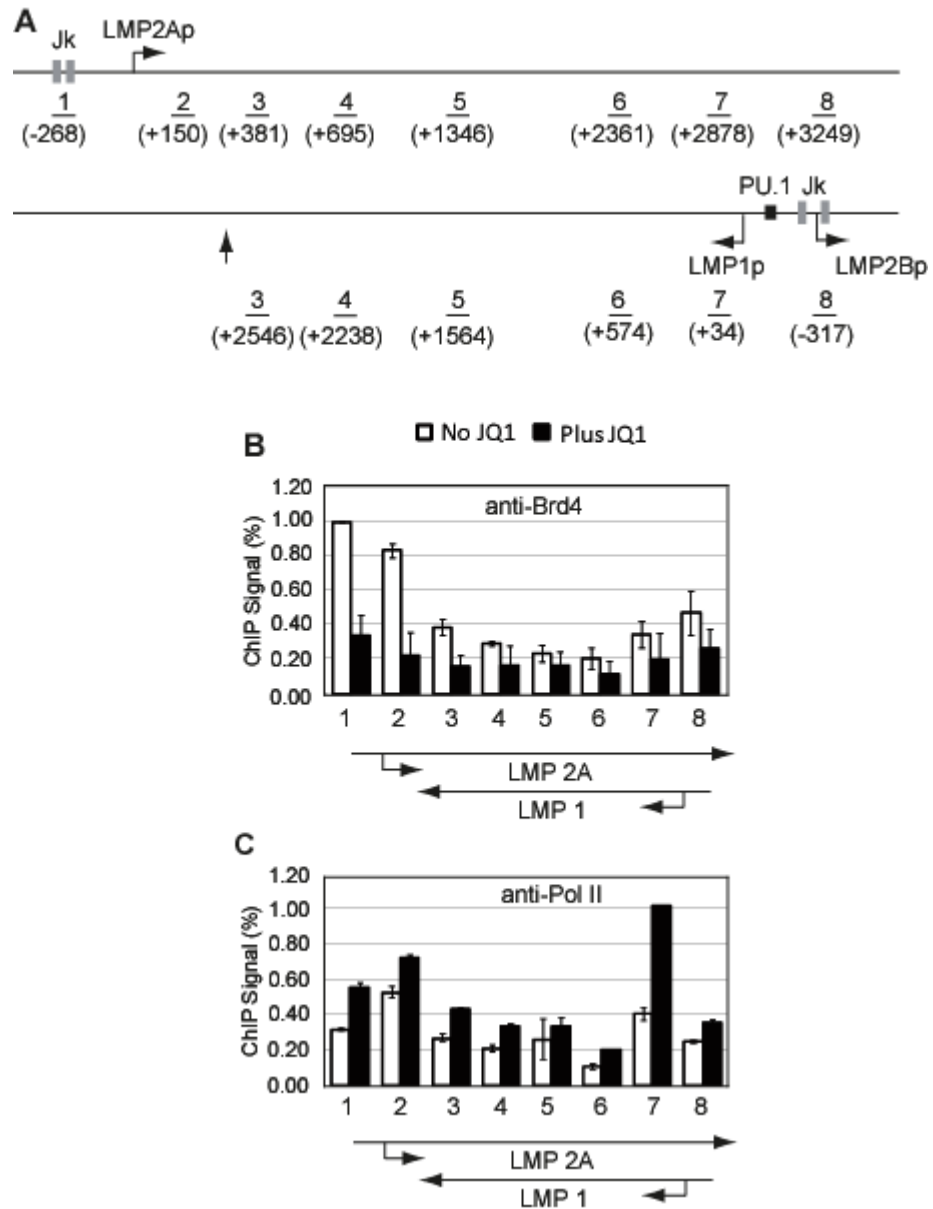
Next we investigated the effects of JQ1 on Brd4 recruitment to the LMP1 and LMP2A promoters (Figure 32). Brd4 occupancy at both LMP1 and 2A promoters was reduced in the presence of JQ1 with a 77% reduction at the LMP 2A promoter (+150 to +231) and a 50% reduction at the LMP1 promoter (-317 to -231). Interestingly Pol II association across the LMP locus was slightly increased upon JQ1 addition (Figure 32B); coinciding with JQ1-dependent increases in LMP 1 transcripts suggesting a possible inhibitory role for Brd4 in LMP 1 transcription. In summary, our data indicate that the binding of Brd4 to Cp is required for Cp



**Figure 30. The Brd4 inhibitor JQ1 inhibits Cp transcription.** Transcription of the Cp-initiated transcripts EBNA 2 and EBNA 1, but not the LMP1 transcript is inhibited when Brd4 binding to chromatin is blocked in the presence of the Brd4 inhibitor JQ1. Mutu III cells were treated with 50 nM JQ1 or DMSO (control) for either (A) 24 hrs (B) 48 hrs and transcript levels determined using specific Q-PCR primers and actin as an endogenous control. Normalised cDNA levels are expressed relative to control samples. Results show the mean  $\pm$  standard deviation of two independent experiments.



**Figure 31. Brd4 inhibition reduces pTEFb recruitment to Cp.** Percentage input signals, after subtraction of no antibody controls, are expressed relative to the highest signal obtained using Cp-specific primers. ChIP with Mutu III cells in the absence (open bars) or presence of 50 nM JQ1 for 48 hrs using anti-Brd4 (n=2, c=1) (A), anti-CDK9 (n=2, c=1) (B), anti-Cyclin T1 (n=2, c=1) (C) and anti-Pol II antibodies (n=2, c=1). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.



**Figure 32. JQ1 increases Pol II recruitment across the LMPp locus.** (A) Diagram of the LMP gene locus showing the locations of the amplicons generated by the indicated primer sets (1-8). Numbers indicate the 5' end of the forward primer relative to the starts of the LMP1 or LMP2A mRNA sequences (bent arrows) in the B95-8 annotated EBV sequence (NC\_007605). The RBP-J $\kappa$  sites (grey boxes) and PU.1 site (black box) are shown. The vertical arrow indicates the position of the LMP1 polyadenylation site. Percentage input signals, after subtraction of no antibody controls, are expressed relative to the highest signal obtained using LMPp-specific primers. ChIP in Mutu III cells in the absence (open bars) or presence of 50 nM JQ1 for 48 hrs using anti-Brd4 (n=2, c=1) (B) and anti-Pol II antibodies (n=2, c=1) (C). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.

transcription since it facilitates the stable association of pTEFb with the stalled polymerases present at Cp.

#### 4.9. Discussion

The requirement for pTEFb to augment transcription at both cellular and viral promoters has been studied at the HIV-1 LTR (Mancebo et al., 1997, Zhu et al., 1997) and genes including p21, MKP-1, c-fos, jun-B and histone H1 (Gomes et al., 2006, Fujita et al., 2009, Fujita et al., 2008, Fujita et al., 2007). Upon release from the inactive complex, pTEFb is free to bind transactivators such as the HIV-1 protein Tat and cellular protein Brd4 (Mancebo et al., 1997, Zhu et al., 1997, Yang et al., 2005, Jang et al., 2005). Brd4 has been shown to recruit pTEFb to promoters that control cell-cycle progression from G1 to S phase (Yang et al., 2008, Mochizuki et al., 2008). Additionally, UV and HMBA stress signals induce the colocalisation of Brd4/pTEFb complexes to inducible genes (Ai et al., 2011a). The interactions between Brd4 and EBNA 1 (Lin et al., 2008) and the requirement for pTEFb in Cp transcription led us to investigate the role of Brd4 at Cp. We showed that Brd4 was recruited to both the C and LMP promoters despite a noticeable absence of pTEFb at LMP1 and 2A. Brd4 associations with pTEFb and acetylated histones have been shown to be weak and transient in nature and require additional factors for stabilized interactions (Yang et al., 2005, Liu et al., 2008). The presence of Pol II stalling may therefore facilitate the association of Brd4-recruited pTEFb to the C promoter by providing high levels of Pol II with which pTEFb can associate.

There is mounting evidence that once recruited to promoters, CDK9 is inhibited as Brd4 prevents the phosphorylation of CDK9 T loop threonine residues and therefore kinase activity (Chen et al., 2004, Ammosova et al., 2011b, Zhou et al., 2009, Li et al., 2005). Only when Brd4 is absent from the template DNA or as CDK9 travels with the elongating Pol II can the threonine residues be phosphorylated and alleviate CDK9 repression (Zhou et al., 2009). Interestingly our ChIP assays showed a sudden surge in Pol II serine 2 CTD phosphorylation that correlates with an 80% reduction in Brd4 occupancy at Cp distal regions (+30kb). Further ChIP experiments at Cp proximal regions using antibodies designed to distinguish between the kinase inactive and active CDK9 forms could determine whether Brd4 regulates CDK9 activity at Cp.

Using the bromodomain inhibitor JQ1, we demonstrated that both pTEFb recruitment and Cp transcription was Brd4-dependent. After Mutu III cells were incubated for 24 hrs with JQ1, there were no substantial differences in either EBNA 2 or EBNA 1 transcripts (Figure 30A). However, at 48 hrs post JQ1 treatment, both sets of transcripts were significantly downregulated (Figure 30B). This discrepancy may be due to the half lives of the EBNA transcripts. Both EBNA 1 and 2 have protein half lives in excess of over 24 hrs (Grasser et al., 1991, Davenport and Pagano, 1999), but not much is known about their mRNA half lives. If they were between 24 and 48 hrs this could explain this discrepancy. Half life experiments of the EBNA proteins in Mutu III cells could be performed to explain this anomaly.

Surprisingly when we treated Mutu III cells with JQ1 we observed an upregulation of LMP1 transcript levels at 24 hrs and Pol II occupancy at 48 hrs (Figures 30 and 32). Similar results have been documented at the E6/E7 promoter during human papillomavirus infection. The viral proteins E6 and E7 once expressed, contribute to oncogenesis by downregulating both the p53 and retinoblastoma (Rb) dependent pathways (Dyson et al., 1989, Scheffner et al., 1990). However the E6/E7 promoter is negatively regulated by another viral protein E2 (Dong et al., 1994). The loss of E2 expression results in the deregulation of E6 and E7 resulting in malignancy (Goodwin and Dimaio, 2000). Studies have shown that E2 functionally competes with pTEFb for the pTEFb interacting domain (PID) of Brd4 (Yan et al., 2010). Once bound to Brd4, E2 recruits Brd4 to the E6/E7 promoter and inhibits transcription by presumably preventing pTEFb-dependent transcription (Wu et al., 2006). These data suggest that LMP1p, like the HPV E6/E7 promoter could be negatively regulated by Brd4. The only way Brd4 has demonstrated transcriptional repression thus far is through the sequestering of pTEFb away from viral proteins such as Tat (Krueger et al., 2010), Tax (Cho et al., 2007, Cho et al., 2010) and E2 (Yan et al., 2010). Since LMP1 requires little if any pTEFb, Brd4 could inhibit LMP1 transcription by preventing various other factors with bromodomains from stimulating transcriptional activation. One candidate could be the ATP-dependent remodeller complex SWI/SNF. Shown to functionally interact with EBNA 2 and through its bromodomain stimulate transcription through the removal of H2A/H2B nucleosomal dimers (Wu et al., 2000a, Chiba et al., 1994, Bruno et al., 2003).



Data from our laboratory has shown a requirement for CDK9-dependent transcription of LMP1 transiently transfected reporter constructs (Bark-Jones et al., 2006). However, here we show Flavopiridol and DRB treatment in Mutu III cells has no effect on LMP 1 transcription. This discrepancy is most likely explained by promoter context. We have demonstrated that the propensity to occlude nucleosomes may stimulate the stalling of Pol II in association with pausing factors and facilitate the recruitment of pTEFb. These Cp-specific mechanisms established *in vivo* may not have occurred in transient assays. Thus pTEFb may be important for Cp and LMP promoter activity in reporter assay systems, but differences in pTEFb requirements are evident in the context of latently infected cells.

Our data also provide the first demonstration of the recruitment of the histone acetyltransferase p300 to the Cp and LMP promoters in the presence of EBNA 2 in Mutu III cells. p300 is a ubiquitously expressed HAT recruited by many transcription factors and was previously shown to interact with EBNA 2 (Wang et al., 2000). Furthermore p300 can also interact with pTEFb, Brd4 and acetylated histones H3 and H4 through acetylated lysine residues (Fu et al., 2007, Huang et al., 2009, Sunagawa et al., 2010). It would be interesting to determine whether the recruitment of pTEFb/Brd4 to Cp is p300-dependent. To examine this, various techniques such as RNAi targeting p300 mRNA (Byun et al., 2009) or p300 knockout cell lines can be utilized (Bundy et al., 2006). Additionally Choi *et al* used a novel p300 histone acetylase inhibitor (HATi) found in green tea called Eigallocatechin-3-Gallate (EGCG) and showed that EBV B cell transformation was inhibited in the presence of EGCG and apoptosis was stimulated (Choi et al., 2009).

EBNA 2 is required for immortalisation and the continued growth and proliferation of lymphoblastoid cell lines (Kempkes et al., 1995b, Cohen et al., 1989). Since the EBNA 2 gene is transcribed from Cp, we analysed both LCL and BL cell viability during treatment with the CDK9 inhibitor Flavopiridol. Proliferation of both cell types was severely inhibited by Flavopiridol treatment (Figures 20 and 21). However, there was a clear difference between the type I and type III BL cell lines. The latency III transcriptional programme is associated with expression of all the EBNAs which augment proliferation and enable cells to be more apoptosis resistant (Gregory et al., 1991). For example EBNA 2 has been shown to functionally prevent Nur77-

induced apoptosis (Lee et al., 2002). LMP1 can upregulate two pro-survival proteins BCL-2 and A20 to prevent activation of the p53-mediated apoptosis pathway (Fries et al., 1996, Okan et al., 1995) and EBNA 3A and 3C can inhibit Bim-mediated apoptosis by preventing its expression (Gavathiotis et al., 2008).

It would be interesting to determine whether the effects of Flavopiridol are mediated in an EBNA 2-dependent manner. However, the only EBV immortalised cells lacking EBNA 2 expression available for these studies are BL cells. Unlike LCLs, both Mutu I and Mutu III cells do not depend on EBNA 2 for their continued growth. Instead the deregulated expression of the c-MYC proto-oncogene drives their continued proliferation (Polack et al., 1996). These results highlight the potential for drug-like derivatives of CDK9 and Brd4 inhibitors as anti-EBV agents. Preliminary experiments could investigate the ability of both Flavopiridol and JQ1 to block EBV immortalisation of B cells *in vitro* and prevent the establishment of LCLs.

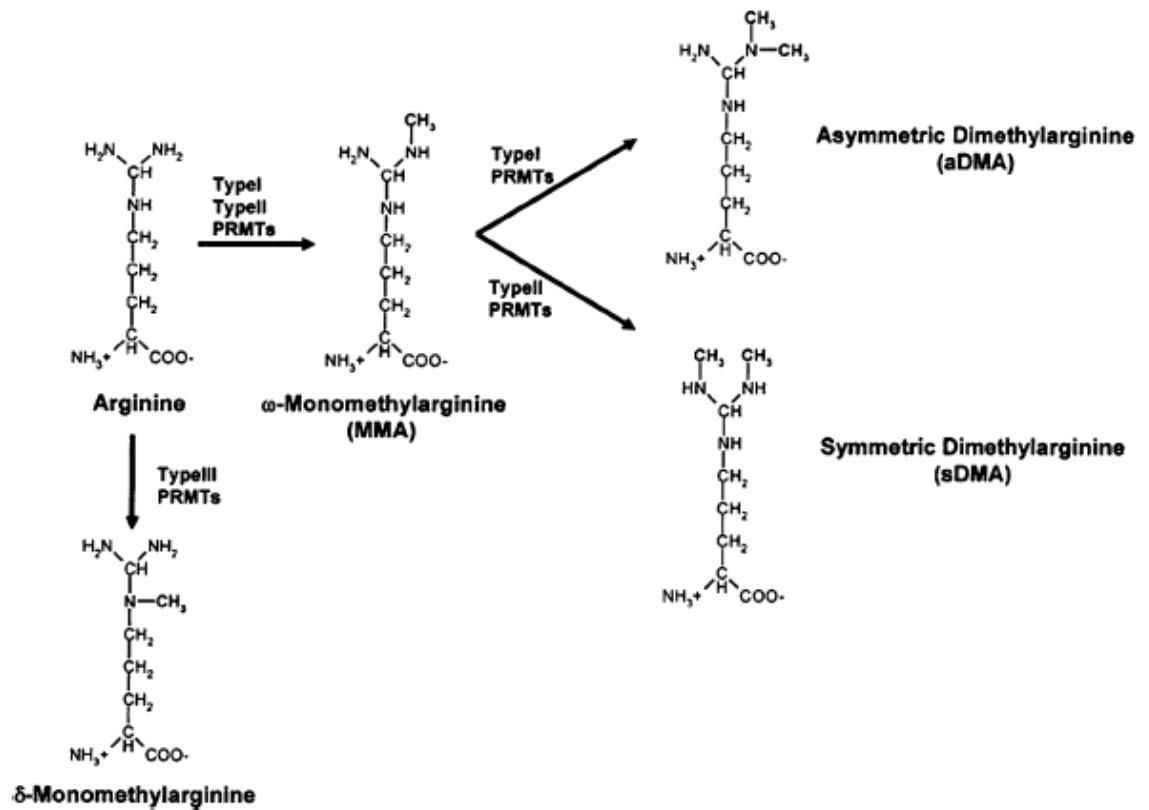
## 5. Investigating the regulation of promoter binding to viral and cellular promoters through methylation of EBNA 2

EBNA 2-mediated transcription can be regulated through post-translational modifications. For example EBNA 2 is a phospho-protein (Grasser et al., 1992), that can be targeted for phosphorylation by CDK1 during mitosis (Yue et al., 2004, Yue et al., 2006) and by the EBV encoded serine/threonine protein kinase PK (Yue et al., 2005). Both target EBNA 2 at serine residue 243 resulting in the inability of EBNA 2 to transactivate the LMP1 promoter and bind to RBPJk. Another potential post-translational mechanism of EBNA 2 is through the methylation of arginine residues, found in the poly-RG region of the protein (amino acids 337 to 354 see Figure 3). Methylation of arginine residues is catalysed by protein arginine methyl transferases (PRMTs), Arginine residues are either mono-methylated (MMA), symmetrically di-methylated (sDMA) or asymmetrically di-methylated (aDMA) (Figure 33). Interestingly *in vivo* studies have shown that sDMA-EBNA 2 is detected and functionally interacts with the SMN protein (Barth et al., 2003, Voss et al., 2001). Further experiments have shown that the deletion of the poly-RG repeat region can prevent the association of EBNA 2 with chromatin and is critical but not essential for B-cell transformation (Tong et al., 1994).

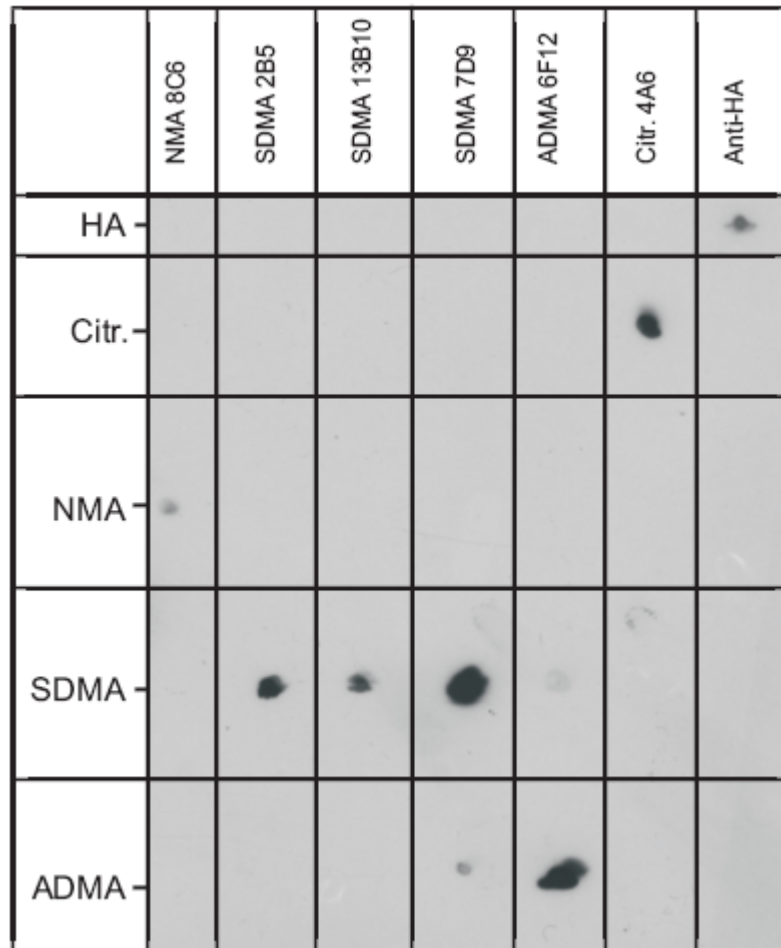
To investigate whether EBNA 2 binding to its target promoters was regulated by its methylation status, in collaboration with Friedrich Grässer's laboratory, we performed ChIP assays using specific monoclonal antibodies that distinguished between both the aDMA and sDMA forms of EBNA 2.

### 5.1. EBNA 2 is methylated in both the sDMA and aDMA orientations *in vivo*

In experiments carried out by Henrik Gross in the Grässer laboratory, mouse and rat monoclonal antibodies were produced using Keyhole Limpet Hemocyanin (KLH) as an immunogen conjugated to various peptide variations of the poly-RG region of EBNA 2. ELISA screening assays with the poly-RG peptides conjugated to Ovalbumin (OVA) demonstrated that



**Figure 33 The methylation of arginine residues by the PRMTs.** Arginine residues can be differentially methylated by the different PRMTs. They can be methylated by type III PRMTs to form δ-Mono-methylarginines or methylated by type I and II PRMTs to form the alternative ω-Mono-methylarginine (MMA). Further methylation to Asymmetric Di-methylarginine (aDMA) by the type I PRMTs or the symmetric Di-methylarginine (sDMA) by type II PRMTs can also occur. Adapted from (Wolf, 2009).

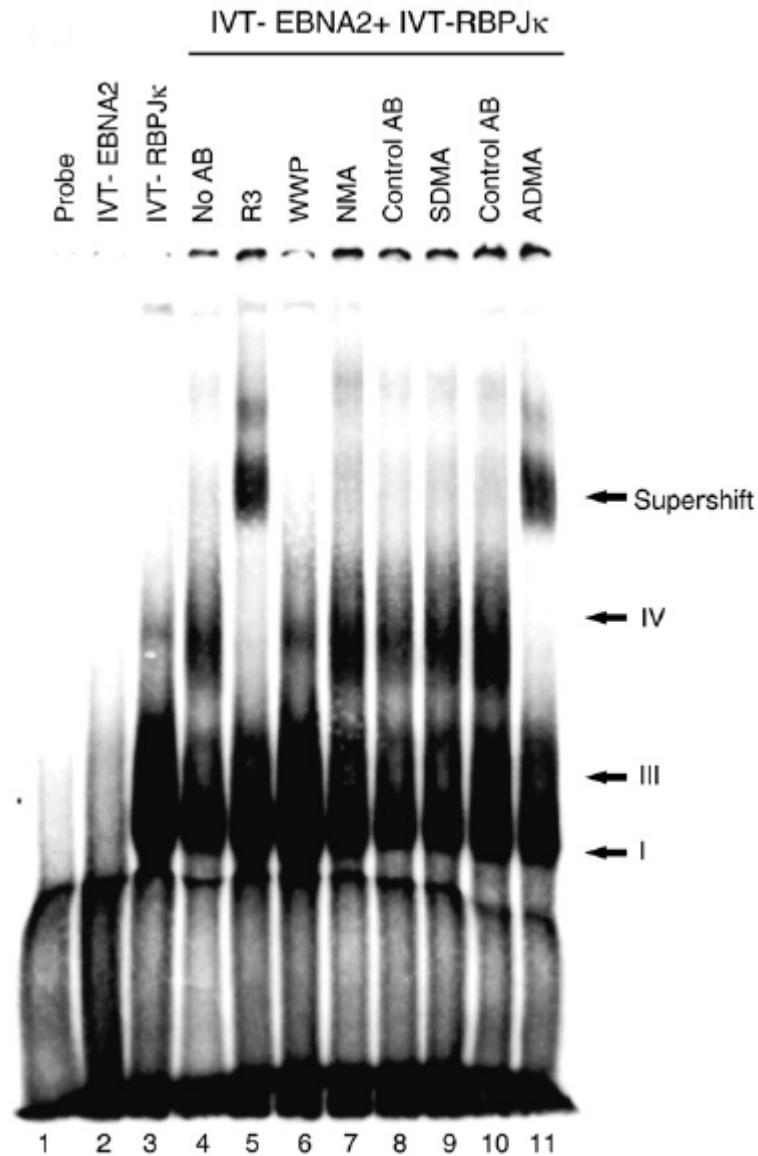


**Figure 34 Characterization of methylation-specific monoclonal antibodies directed against the RG-repeat of EBNA2 by dot-blot analysis.** The OVA-coupled peptides containing nonmethylated (NMA)-, symmetrically dimethylated (sDMA), asymmetrically dimethylated (aDMA) Arginines or citrulline instead of Arginine residues were spotted onto nitrocellulose strips. OVA-HA conjugate served as internal control and was detected by the HA-specific antibody 3F10 (Roche, Penzberg, Germany). Each strip was incubated with the indicated antibody, and bound antibody was visualized by ECL using peroxidase-coupled secondary anti-rat or anti-mouse antibody. Experiment was performed by Henrik Gross.

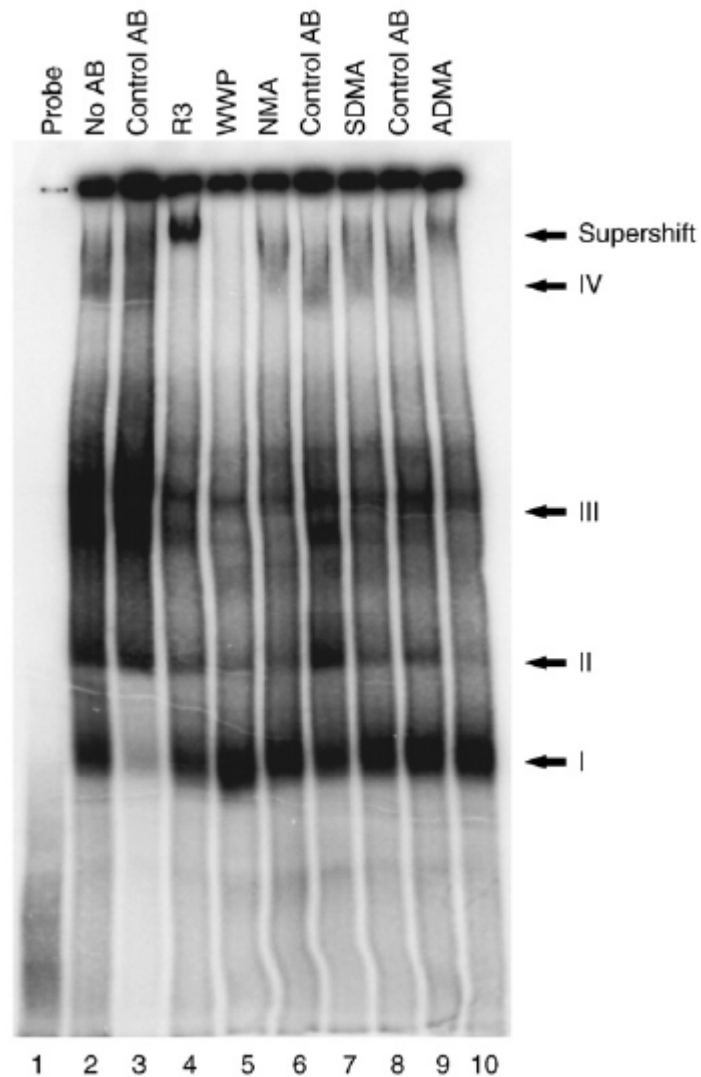
3 sDMA antibodies and an aDMA antibody generated reacted against their cognate peptides with minimal cross-reactivity (Figure 34). Previously EBNA 2 was shown to contain methylated motifs in the Poly-RG region *in vivo* (Barth et al., 2003, Voss et al., 2001). To determine if EBNA 2 contained either an sDMA or aDMA modification, Electro-mobility shift assays (EMSA) were carried out. *In vitro* transcribed EBNA 2 and RBPJk were incubated with probe containing two RBPJk binding sites and either EBNA 2 methylation specific antibodies (NMA, sDMA and aDMA), methylation independent EBNA 2 antibody R3 (Kremmer et al., 1995) or antibodies that disrupted the EBNA 2/RBPJk interaction WWP, (Sauder et al., 1994). Figure 35 shows that the WWP, NMA and sDMA-EBNA 2 (lanes 6, 7 and 9) all failed to bind to the DNA complexes. However, the R3 and aDMA-EBNA 2 antibodies (lanes 5 and 11) could both bind to DNA complexes causing a supershift (Figure 35). Further experiments using the BL Raji cell line which contain a deletion in the EBNA 3C gene, were incubated with the probe and the same antibodies (Figure 36). Similarly, the WWP, NMA and sDMA-EBNA 2 antibodies failed to bind to the DNA (lanes 5, 6 and 8). Both the R3 (lane 4) and aDMA-EBNA2 (lane 10) again caused a supershift (Figure 36). These data indicate that aDMA -EBNA 2 has the capacity to bind DNA *in vitro*.

## 5.2. Analysis of sDMA-EBNA 2 binding *in vivo*

To determine whether aDMA-EBNA 2 was present at promoters *in vivo*, we carried out ChIP assays in Mutu I and Mutu III cells (Figure 37). Using the methylation-independent monoclonal rat antibody R3, we confirmed that EBNA 2 was bound to Cp at the upstream RBPJk site as previously shown using the mouse monoclonal antibody to EBNA 2 (PE2) (Figures 37A and B). The aDMA-EBNA 2 antibody was associated with Cp and displayed the same binding profile as observed using the R3 and PE2 antibodies (Figure 37C). Interestingly, no signal was detected at Cp when EBNA 2 was precipitated using the sDMA rat antibody (13B10) (Figure 37D). It is therefore possible that sDMA-EBNA 2 does not bind *in vivo* or that the sDMA antibody does not precipitate sDMA-EBNA 2 under ChIP conditions. Experiments were therefore carried out to determine whether the sDMA antibody 13B10 could immunoprecipitate EBNA 2 under ChIP conditions. Western blot analysis of eluted immune complexes from ChIP revealed that no

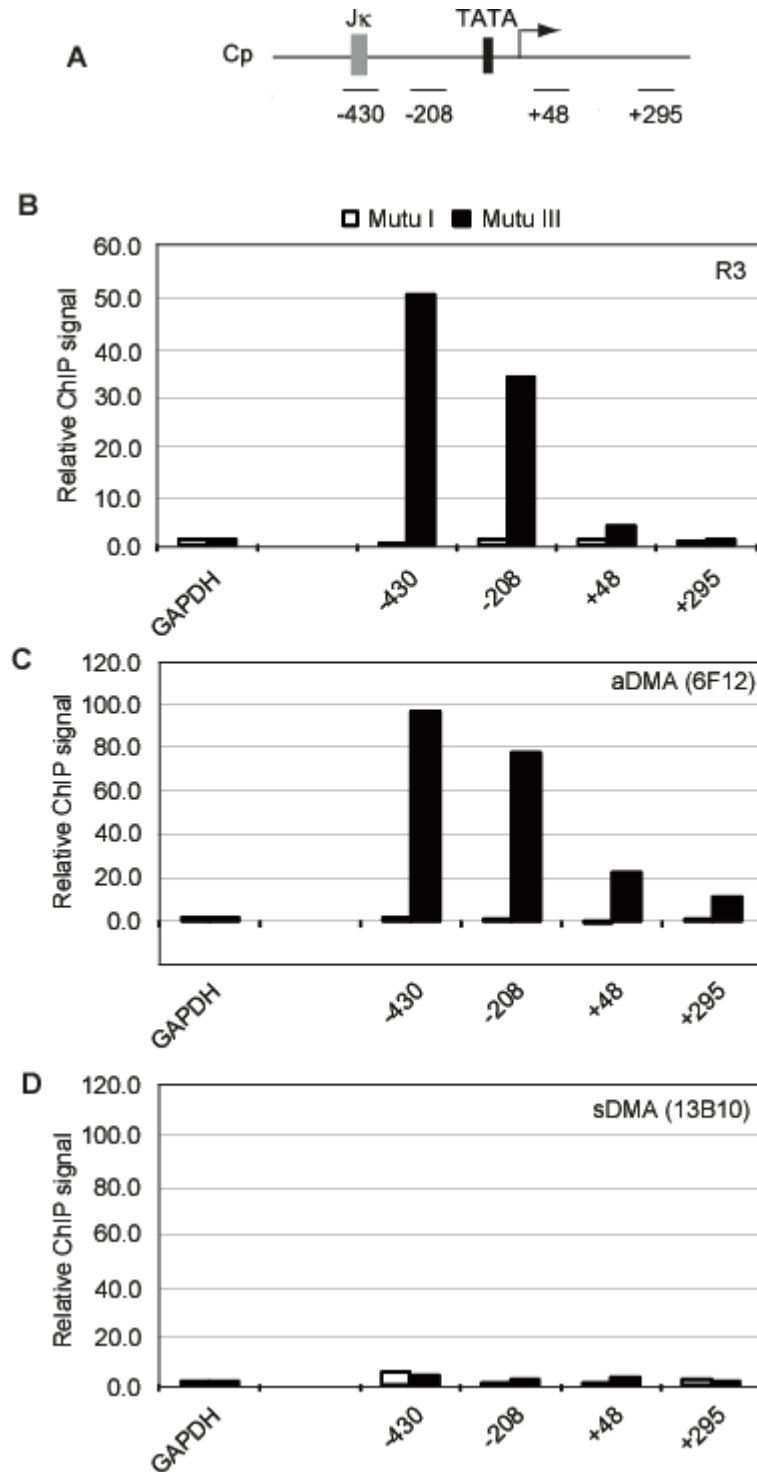


**Figure 35 aDMA-modified EBNA 2 is present in DNA-binding complexes.** EBNA2 and RBP-Jk synthesized in a coupled *in vitro* transcription-translation system derived from rabbit reticulocytes (Promega) were used in a gel shift assay (EMSA) (Meitinger et al., 1994) after preincubation of the proteins with the indicated antibodies. Antibodies used are indicated above each lane. The complexes III and IV which are formed by RBP-Jk and RBP-Jk plus EBNA 2, respectively, as well as complex IV supershifted with mAb R3 (lane 4) or aDMA-specific mAb 6F12 (lane 11) are indicated. Control antibodies corresponded to the respective IgG-subtype of each antibody. The arrow points at the EBNA2-containing complex IV that is supershifted by R3 and aDMA-6F12 but destroyed by WWP-6C8. Experiments were performed by Henrik Gross.

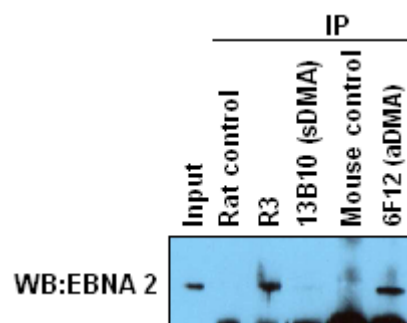


**Figure 36 aDMA-modified EBNA 2 is present in DNA-binding complexes in Raji cells.** EBNA2-containing Raji cell extracts were incubated with the indicated antibodies and then assayed in a gel shift assay. The complexes III and IV which are formed by RBP-Jk and RBPJk plus EBNA 2, respectively, as well as complex IV supershifted with mAb R3 (lane 4) or aDMA-specific mAb 6F12 (lane 10) are indicated. Control antibodies corresponded to the respective IgG-subtype of each antibody. Experiments were performed by Henrik Gross.





**Figure 37 aDMA-EBNA 2 binds Cp in Mutu III cells.** (A) Diagram showing the locations of the amplicons generated by the indicated primer sets (Appendix D) at the C promoter. Numbers indicate the 5' end of the forward primer relative to the Cp transcription start site in the annotated B95-8 EBV sequence (NC\_007605.1). The RBP-J $\kappa$  site (grey box) and TATA box (black box) are shown. Results show one experiment carried out using chromatin from Mutu I cells (open bars) and Mutu III cells (black bars). Percentage input signals, after subtraction of no antibody controls are expressed for comparison purposes relative to GAPDH primers. ChIP using anti R3-EBNA 2 (B), anti aDMA-EBNA 2 (C) and anti sDMA-EBNA 2 antibodies (D).



**Figure 38. Antibody 13B10 (sDMA-EBNA 2) does not immunoprecipitate under ChIP conditions.** The different methylated forms of EBNA 2 (sDMA and aDMA) were immunoprecipitated (IP) using both protein A and protein G sepharose beads from Mutu III cells. Western blots (WB) probed IPs and species specific controls for EBNA 2

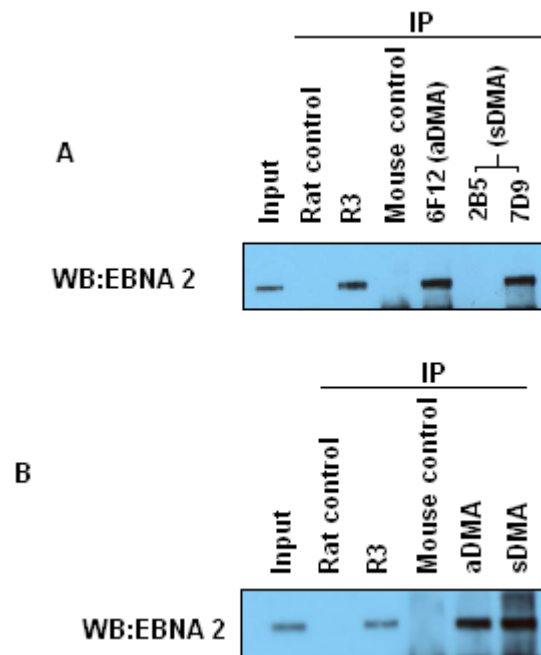
sDMA-EBNA 2 was precipitated, although EBNA 2 was efficiently IP'd using the R3 and 6F12 aDMA antibody (Figure 38). The lack of detectable Cp DNA binding in ChIP assays carried out using the 13B10 sDMA-EBNA 2 antibody can therefore be attributed to the failure of the antibody to precipitate EBNA 2.

### **5.3. The differential methylation of EBNA 2 regulates its binding to viral promoters**

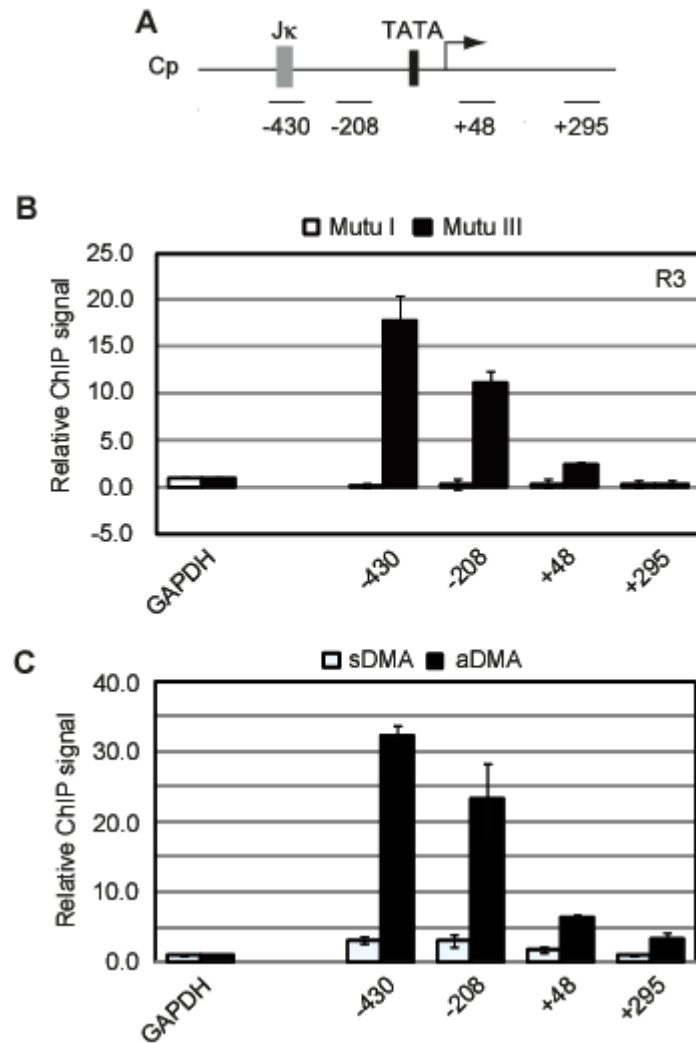
The failure of the 13B10 antibody to precipitate EBNA 2 under ChIP conditions, led us to test the 2B5 and 7D9 mouse monoclonal antibodies. ChIP was initially carried out in the absence of cross-linking to test their ability to precipitate EBNA 2 in ChIP buffers. Western blot analysis demonstrated that the 7D9 but not the 2B5 precipitated sDMA-EBNA 2 (Figure 39A). Further experiments confirmed that the 7D9 antibody was also able to precipitate sDMA-EBNA 2 from crosslinked chromatin extracts at a comparable level to the aDMA (6F12) antibody (Figure 39B). ChIP-QPCR assays detected high-level association of aDMA-EBNA 2 with Cp in Mutu III cells but sDMA-EBNA 2 was only detectable at Cp at low levels despite both antibodies precipitating equal amounts of EBNA 2 (Figure 40C). To test if this profile was unique to Cp, we next examined the LMP gene locus for association with the aDMA and sDMA forms of EBNA 2. Similar to Cp, we detected aDMA-EBNA 2 at high-levels at both the LMP1 and LMP2A promoters compared to sDMA-EBNA 2 (Figure 41). In conclusion these data reveal how the differential methylation of the poly-RG region of EBNA 2 regulates its ability to interact with viral promoters and transactivate them.

### **5.4. aDMA-EBNA 2 binds to the CD23 promoter to activate transcription**

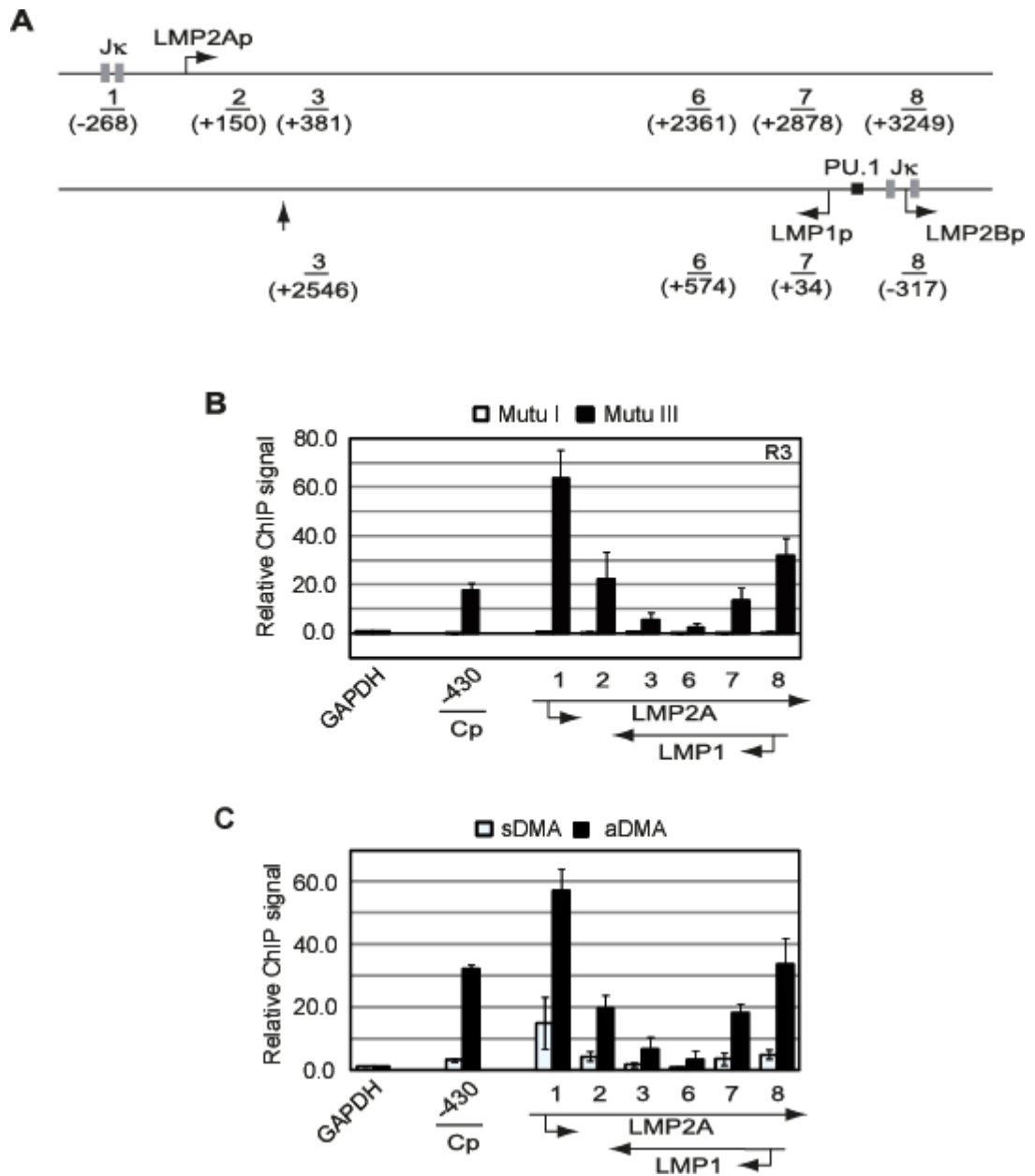
In addition to its role as an activator of the viral promoters C, LMP1, 2A and 2B, EBNA 2 has also been shown to activate cellular genes such as CD21 (Aman et al., 1990), CD23 (Wang et al., 1987a) and Cyclin D2 (Sinclair et al., 1994). We therefore investigated whether arginine methylation regulated EBNA 2 association with cellular gene promoters. We chose to investigate CD23 because of its mapped EBNA 2 response elements, unlike CD21 whose activation by EBNA 2 is still unclear.



**Figure 39. Antibodies 6F12 and 7D9 targeting EBNA 2 Immunoprecipitates in Mutu III cells under ChIP conditions.** The different methylated forms of EBNA 2 (sDMA and aDMA) were immunoprecipitated (IP) using both protein A and protein G sepharose beads (A) in the absence of crosslinked chromatin where formaldehyde was not added or (B) crosslinked chromatin from Mutu III cells. Western blots (WB) probed IPs and species specific controls for EBNA 2.



**Figure 40. sDMA-EBNA 2 does not bind Cp in Mutu III cells.** (A) Diagram showing the locations of the amplicons generated by the indicated primer sets (Appendix D) at the C promoter. Numbers indicate the 5' end of the forward primer relative to the Cp transcription start site in the annotated B95-8 EBV sequence (NC\_007605.1). The RBP-J $\kappa$  site (grey box) and TATA box (black box) are shown. Percentage input signals, after subtraction of no antibody controls, are expressed relative to GAPDH. (B) ChIP in Mutu I cells (open bars) and Mutu III cells (black bars) using anti-R3 EBNA 2 (n=3, c=3). (C) ChIP in Mutu III cells using anti-aDMA-EBNA 2 (n=5, c=5) (black bars) and anti sDMA-EBNA 2 (open bars) antibodies (n=3, c=2). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.



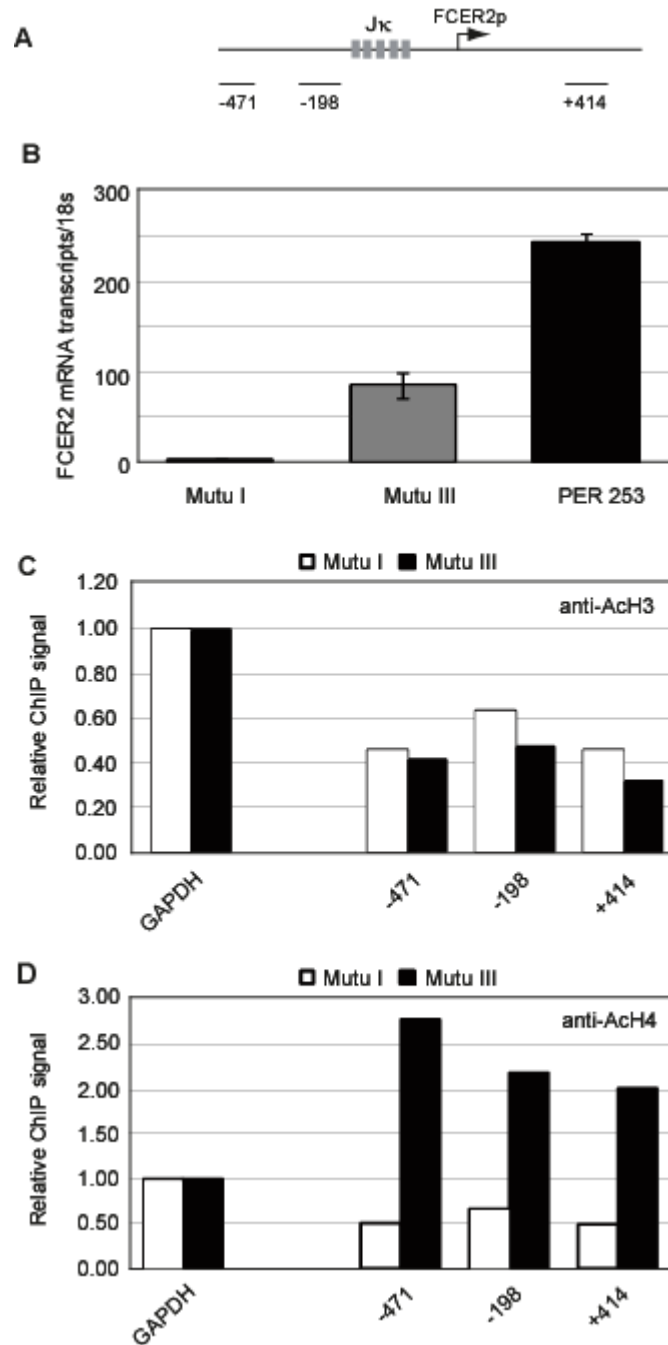
**Figure 41. aDMA-EBNA binds LMP gene locus in Mutu III cells.** (A) Diagram of the LMP gene locus showing the locations of the amplicons generated by the indicated primer sets (1-3 and 6-8). Numbers indicate the 5' end of the forward primer relative to the starts of the LMP1 or LMP2A mRNA sequences (bent arrows) in the B95-8 annotated EBV sequence (NC\_007605). The RBP-J $\kappa$  sites (grey boxes) and PU.1 site (black box) are shown. The vertical arrow indicates the position of the LMP1 polyadenylation site. Percentage input signals, after subtraction of no antibody controls, are expressed relative to GAPDH. (B) ChIP in Mutu I cells (open bars) and Mutu III cells (black bars) using anti-R3 EBNA 2 (n=5, c=3). (C) ChIP in Mutu III cells using anti-aDMA-EBNA 2 (n=3, c=3) (black bars) and anti-sDMA-EBNA 2 (open bars) antibodies (n=3, c=2). Where n denotes the number of experimental repeats and c denotes the number of chromatin batches.

CD23 is a transmembrane protein which acts as a low affinity IgE receptor (Suter et al., 1987, Ludin et al., 1987). CD23 can either be membrane bound or cleaved from the cell surface to form soluble CD23 (sCD23). Activated CD23 augments escape from apoptosis and promotes the proliferation of B cells (White et al., 1997, Liu et al., 1991, Cairns and Gordon, 1990). Located on chromosome 19, the CD23 gene (FCER2) contains 5 potential RBPJk sites situated between -171 and -35 from the transcriptional start site (Chang et al., 2005). However it appears the canonical GTGGGAA sequence located between nucleotides -171 and -165 is the most critical for both EBNA 2 and Kaposi's Sarcoma-associated viral protein RTA to activate CD23 transcription (Ling et al., 1994, Chang et al., 2005).

Initially we compared Mutu I, Mutu III and a LCL to determine whether CD23 transcript levels were indeed higher in EBNA 2 expressing cells. Figure 42B confirmed that EBNA 2 expressing cells did upregulate CD23 significantly higher than the non-EBNA 2 expressing control. We also investigated histone acetylation patterns that encompassed the CD23 promoter (Figures 42C and D). Interestingly, the CD23 promoter showed no significant differences in AcH3 between Mutu I and Mutu III cells. However, AcH4 levels were substantially higher in EBNA 2 expressing Mutu III cells. ChIP assays next confirmed that the peak of EBNA 2 binding at Cp was between nucleotides -198 and -87 in Mutu III cells (Figure 43B). In line with our previous results, we demonstrate that aDMA-EBNA 2 has an enhanced promoter binding preference over sDMA-EBNA 2 (Figure 43C). These data indicate that aDMA-EBNA 2 preferentially associates with both viral and cellular genes to augment transcription.

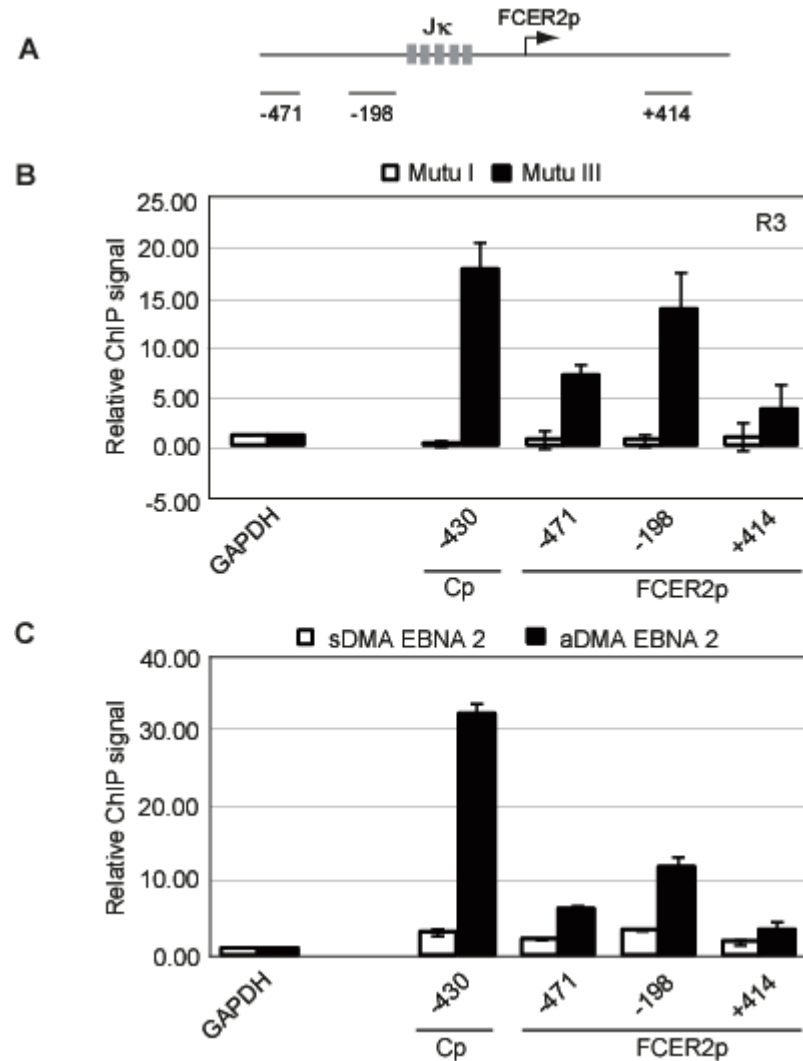
### **5.5. aDMA EBNA 2 binds to the CD79B promoter to downregulate transcription**

To determine whether arginine methylation also regulates EBNA 2 association with repressed gene targets, we examined the repression of CD79B. CD79B, also known as B29 or Ig $\beta$ , is expressed in all stages of B cell development (Hermanson et al., 1988, Benlagha et al., 1999) and forms a heterodimer as part of the B-cell antigen receptor complex (BCR) with CD79A (Hombach et al., 1990). Both CD79A and CD79B are required for BCR cell surface expression and signaling leading to B cell activation (Clark et al., 1992, Grupp et al., 1993).



**Figure 42. The CD23 promoter is active in Mutu III cells.** (A) Diagram of the CD23 gene locus showing the locations of the amplicons generated by the indicated primer sets (Appendix D). Numbers indicate the 5' end of the forward primer relative to the start of the CD23 mRNA sequence (bent arrows) on chromosome 19. The 5 RBP-J $\kappa$  sites (grey boxes) are shown. (B) PCR amplification of CD23-specific transcripts using Mutu I, Mutu III and the PER 253 LCL. Results show the mean  $\pm$  standard deviation of two independent experiments carried out in duplicates. (C) Results show one experiment carried out on chromatin from Mutu I cells (open bars) and Mutu III cells (black bars). Percentage input signals, after subtraction of no antibody controls, are expressed using GAPDH-specific primers. ChIP using anti-AcH3 and (C) anti-AcH4 antibodies. (D).



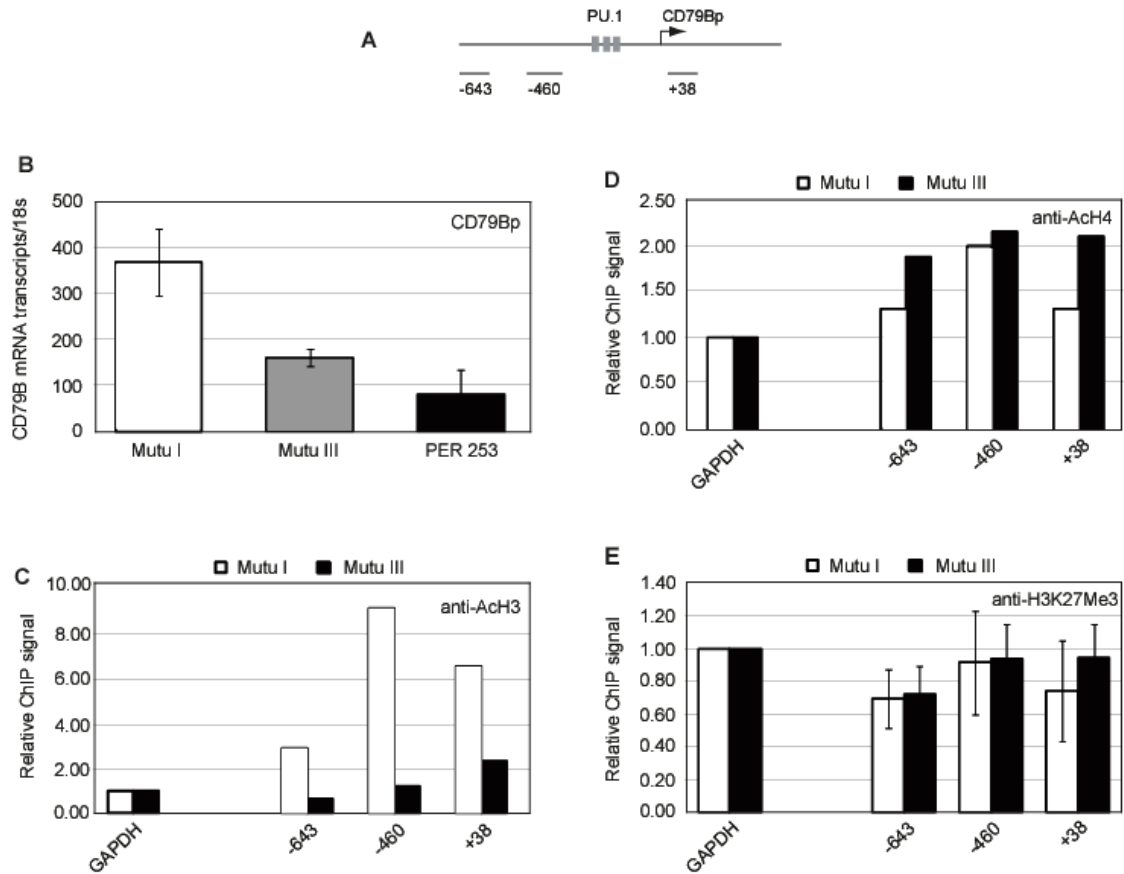


**Figure 43 aDMA-EBNA binds and activates the CD23 promoter in Mutu III cells.** (A) Diagram of the CD23 gene locus showing the locations of the amplicons generated by the indicated primer sets. Numbers indicate the 5' end of the forward primer relative to the start of the CD23 mRNA sequence (bent arrows) on chromosome 19. The 5 RBP-J $\kappa$  sites (grey boxes) are shown. Results show the mean  $\pm$  standard deviation of two independent experiments carried out using two different chromatin batches from Mutu I cells (open bars) and Mutu III cells (black bars). Percentage input signals, after subtraction of no antibody controls, are expressed using GAPDH-specific primers. ChIP using anti-R3 EBNA 2 (B) anti-aDMA-EBNA 2 and anti sDMA-EBNA 2 antibodies (C).

Further studies have shown B cell development and maturation are dependent on both CD79A and CD79B, in particular VDJH recombination (Torres et al., 1996, Papavasiliou et al., 1995, Gong and Nussenzweig, 1996, Hermanson et al., 1988). The CD79B gene (B29) located on chromosome 17, contains a TATA-less promoter and therefore transcription initiates from four different start sites (Hermanson et al., 1989, Omori and Wall, 1993). Moreover the CD79A gene (mb1) also is TATA-less and shares a high homology for transcription factor binding sites with B29 (Travis et al., 1991). Interestingly Mori et al showed that upon EBV infection in SCID mice both CD79A and CD79B were significantly downregulated (Mori et al., 1994). More recently two independent microarrays using either LCLs and EBV negative BL cells conditionally expressing LMP1 or EBNA 2 both confirmed CD79B as a target for downregulation (Maier et al., 2006, Cahir-Mcfarland et al., 2004). Further experiments have shown that LMP1 downregulates both CD79B and CD79A in germinal centre B cells (Vockerodt et al., 2008). Additionally in Chronic Lymphocytic Leukaemia (CLL) patients CD79B expression on the cell surface is greatly reduced (Thompson et al., 1997).

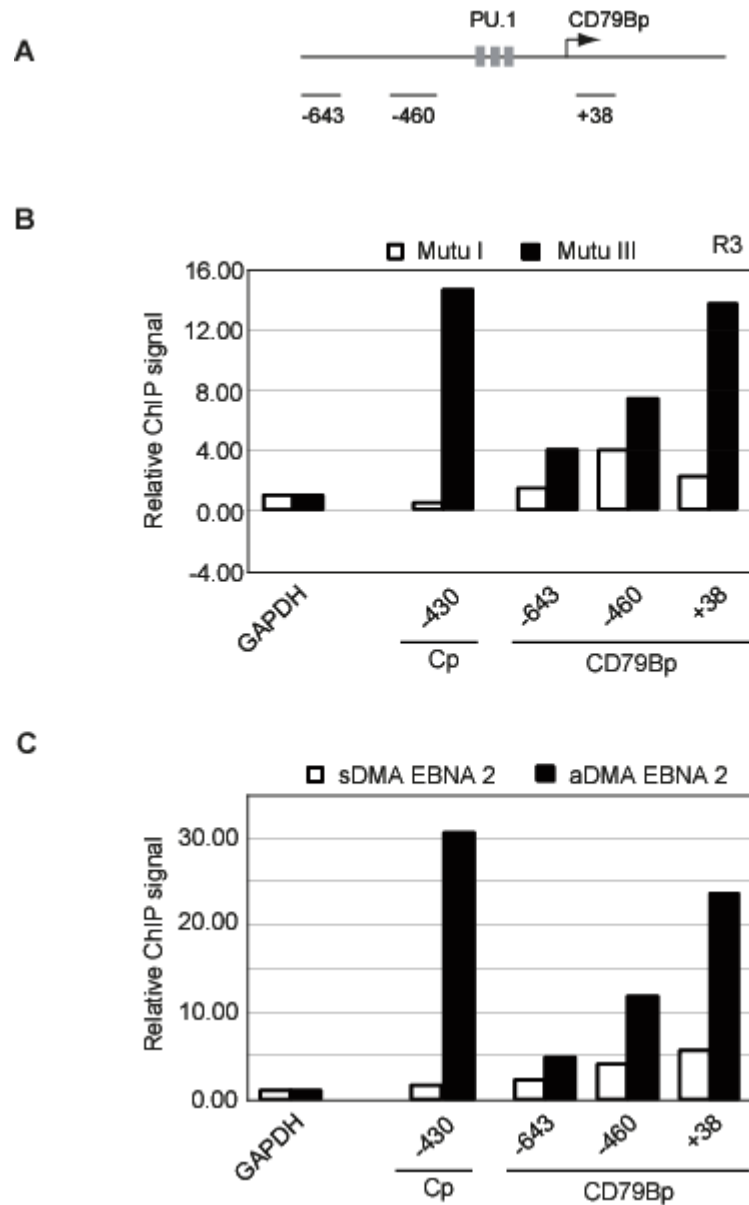
To investigate the expression levels of CD79B in Mutu I and Mutu III cells, we designed specific primers that would amplify all 3 potential isoforms. As expected, we detected reduced expression of CD79B in Mutu III cells and an LCL that expresses EBNA 2 and LMP1, when compared to Mutu I cells (Figure 44B). Next we investigated histone modifications at the CD79B promoter using ChIP. CD79Bp was highly acetylated on histone H3 in Mutu I cells compared to Mutu III cells, consistent with the level of gene expression, with no difference in AcH4 levels (Figures 44C and D). Further experiments examined CD79Bp for H3K27me3, a known gene silencing mark associated with the activity of the PRC (for review see Chase and Cross, 2011). However, we did not detect any differences in H3K27me3 status between Mutu I and Mutu III cells (Figure 44E), indicating that repression was possibly mediated by polycomb-independent mechanisms.

Promoter studies have shown there is no RBP-Jk binding site, instead EBNA 2 adapter protein PU.1 can bind B29 between nucleotides -66 and -100 from the predominant transcriptional start site (Omori and Wall, 1993). Therefore we investigated whether EBNA 2



**Figure 44 The CD79B promoter is repressed Mutu III cells.** (A) Diagram of the CD79B gene locus showing the locations of the amplicons generated by the indicated primer sets. Numbers indicate the 5' end of the forward primer relative to the start of the CD79B mRNA sequence (bent arrows) on chromosome 17. The 3 potential PU.1 sites (grey boxes) are shown. (B) PCR amplification of CD79B-specific transcripts using Mutu I, Mutu III and the PER 253 LCL. Results show the mean  $\pm$  standard deviation of a two independent experiments carried out in duplicates. (C) Results show one experiment from Mutu I cells (open bars) and Mutu III cells (black bars). Percentage input signals, after subtraction of no antibody controls, are expressed using GAPDH-specific primers. ChIP using anti-AcH3 and (D) anti-AcH4 antibodies. (E) Results show the mean  $\pm$  standard deviation of two independent ChIP experiments carried out using two different chromatin batches from Mutu I cells (open bars) and Mutu III cells (black bars) using the anti-H3K27Me3 antibody.

could directly bind and downregulate CD79Bp in Mutu III cells and decipher which methylated form of EBNA 2 was required for this downregulation. Our ChIP assays demonstrated the binding of EBNA 2 to CD79Bp (Figure 45B). Further experiments showed the enhanced binding of aDMA-EBNA 2 when compared to the sDMA-EBNA 2 (Figure 45C). These data suggest that the differential methylation of EBNA 2 regulates its transcriptional activity at both viral and cellular genes by altering the affinity to which it interacts with adaptor proteins.



**Figure 45 aDMA-EBNA binds and represses the CD79B promoter in Mutu III cells.** (A) Diagram of the CD79B gene locus showing the locations of the amplicons generated by the indicated primer sets. Numbers indicate the 5' end of the forward primer relative to the start of the CD79B mRNA sequence (bent arrows) on chromosome 17. The 3 PU.1 sites (grey boxes) are shown. Results show one experiment from Mutu I cells (open bars) and Mutu III cells (black bars). Percentage input signals, after subtraction of no antibody controls, are expressed using GAPDH-specific primers. ChIP using anti-R3 EBNA 2 (B), anti-aDMA-EBNA 2 and anti sDMA-EBNA 2 antibodies (C).

## 5.6. Discussion

It has been previously documented that EBNA 2 binding to DNA through adapter proteins is inhibited by hyperphosphorylation (Yue et al., 2004, Yue et al., 2006). Here we show EBNA 2 is also a target for methylation in the poly-RG repeat region and that this mediates the ability of EBNA 2 to associate with DNA. The asymmetrically di-methylated form of EBNA 2 (aDMA-EBNA 2) was shown to bind viral promoters Cp, LMP1 and LMP2A to augment their activation, whereas the symmetric di-methylated form (sDMA-EBNA 2) bound at lower levels. Additionally we examined two cellular genes CD23 and CD79B and found that only aDMA-EBNA 2 could bind cellular gene promoters efficiently. Interestingly, the poly-RG (amino acids 337 to 354) and CR6 (amino acids 318 to 327) regions are in close proximity. The CR6 region of EBNA 2 is critical for DNA interactions through the associations with RBP-Jk and PU.1 (Grossman et al., 1994, Henkel et al., 1994, Johannsen et al., 1995). Therefore the sDMA-EBNA 2 may cause conformational changes, preventing associations between RBP-Jk/PU.1 and two key EBNA 2 tryptophan residues W323 and W324 (Ling et al., 1993).

The binding of the survivor motor neuron protein (SMN) to sDMA-EBNA 2 has been shown to stimulate LMP1 transcription (Barth et al., 2003, Voss et al., 2001). We carried out additional ChIP assays to determine whether SMN was located at LMP1p, to potentially augment transcription through the assembly of spliceosome components (Barth et al., 2003). Although, SMN clearly immunoprecipitated in Mutu III cells, ChIP assays could not detect any SMN at Cp or LMP1p (data not shown). Since our ChIP assays did detect some sDMA-EBNA 2 at promoters, SMN could sequester sDMA-EBNA 2 away from these regions allowing the binding of aDMA-EBNA 2 to promoters. It is also plausible that sDMA-EBNA 2 could functionally compete with aDMA-EBNA 2 to prevent transcriptional activation.

Interestingly, *in vitro* gel shift experiments were only supershifted by complexes containing total EBNA 2 (R3) or by the aDMA-EBNA 2 form. However, ChIP data suggested that both the aDMA and sDMA forms of EBNA 2 could interact with DNA. EMSA experiments investigating the interactions between RBP-Jk and EBNA 2 resulted in the subsequent binding and dissociation of EBNA 2/RBP-Jk complexes from DNA (Waltzer et al., 1994). This may

demonstrate a requirement for other factors that stabilise these interactions. One possible protein responsible for stabilisation could be SKIP, which interacts with both EBNA 2 and RBP-Jk proteins (Zhou et al., 2000a). Another important factor could be the absence of chromatinized DNA. The presence of histones mediating key interactions or preventing access to regulatory sites may contribute to regulate transcription.

Arginine methylation of proteins by the PRMTs can have both positive and negative permutations on transcription. For example arginine histone methylation of H3R2me2a by PRMT6 can inhibit transcriptional activation (Iberg et al., 2008, Hyllus et al., 2007). Whereas H2AR3me2a is associated with active promoters (Li et al., 2010). The sDMA of IL-2 in T cells by PRMT5 results in the activation of IL-2 dependent genes (Richard et al., 2005). Moreover the aDMA of STAT-1 by PRMT1 is also associated with activation of transcription (Mowen et al., 2001). Either aDMA or sDMA modifications can be inhibitory or stimulatory depending on the protein.

We also investigated the role of EBNA 2 methylation in the activation and repression of cellular genes. A well characterised cellular target gene upregulated by EBNA 2 is CD23 (Wang et al., 1987a). CD23 transcripts can be detected 36 hrs post EBV infection (Alfieri et al., 1991). CD23 is a transmembrane protein which acts as a low affinity IgE receptor (Suter et al., 1987, Ludin et al., 1987). CD23 can either be membrane bound or cleaved from the cell surface to form soluble CD23 (sCD23). Activated CD23 augments escape from apoptosis and promotes the proliferation of B cells (White et al., 1997, Liu et al., 1991, Cairns and Gordon, 1990). We demonstrated the upregulation of CD23 transcripts through the binding of aDMA-EBNA 2, this may be an Ach3-independent activation because Ach3 levels at CD23p in Mutu I and Mutu III were unchanged. It would be interesting to investigate how EBNA 2 upregulates CD23 expression in more detail. ChIP assays at the CD23 promoter and subsequently downstream could determine whether elongation factors such as pTEFb or DSIF are recruited in an EBNA 2 dependent manner.

CD79B is ubiquitously expressed throughout B cell maturation and is involved in B cell activation and BCR signalling (Torres et al., 1996, Papavasiliou et al., 1995, Gong and Nussenzweig, 1996, Hermanson et al., 1988, Clark et al., 1992). The CD79B gene is found to

be downregulated in EBV transformed LCLs, conditional B cells expressing EBNA 2 and CLL (Thompson et al., 1999, Cahir-Mcfarland et al., 2004, Maier et al., 2006). This downregulation would normally result in apoptosis in germinal centre B cells, however EBV has been shown to rescue these defective B cells from apoptosis (Mancao et al., 2005, Chaganti et al., 2005, Bechtel et al., 2005). Here we show the binding of aDMA-EBNA 2 and subsequent downregulation of CD79B in Mutu III cells. In addition, this downregulation was independent of the polycomb repressive complexes and ACh4. As far as we are aware this is the first time EBNA 2 has been shown to bind upstream of a gene that is known to be downregulated. Furthermore the ability of LMP1 to downregulate CD79Bp independently of EBNA 2 (Vockerodt et al., 2008), suggests a potential co-repressive mechanism may exist. EBNA 2 may directly bind and inhibit CD79Bp transcription and indirectly inhibit CD79Bp through the upregulation of LMP1p and cellular signalling.

Interestingly the B cell specific transcriptional co-activator OCA-3/OBF-1/Bob1 has been shown to activate CD79Bp and reactivate transcription in GH3 pituitary cells (Malone et al., 2006, Malone and Wall, 2002, Kim et al., 1996). ChIP-seq carried out in Mutu III cells by Michael McClellan in our laboratory revealed a potential EBNA 2 binding site just upstream of Bob1p coinciding with an RBPJk site (data not shown). Through the downregulation of Bob1, EBNA 2 could ensure that the reactivation of CD79Bp is prevented, highlighting another potential mechanism for EBNA 2-mediated CD79Bp repression. Initially, the potential EBNA 2 binding site at the Bob1 promoter will need to be verified through ChIP.

In summary our data demonstrates that the differential methylation of the EBNA 2 poly-RG region is required to regulate its binding and transcriptional regulation of viral and cellular promoters. EBNA 2 in its aDMA form appears to have a higher binding affinity at promoters than its sDMA counterpart. Identifying the PRMTs responsible for EBNA 2 methylation may provide potential therapeutic targets for EBV-associated diseases such as IM and PTLD.



## 6. Discussion

The EBV Cp encodes a long transcript of ~120 kb in length that is differentially spliced and critical for the immortalisation of B cells. Therefore the transcription factors and other mechanisms used to regulate Cp are of utmost importance in EBV pathogenesis. Moreover, EBNA 2 is the main latent viral transcription factor, responsible for the regulation of all latency III protein-encoding viral promoters (including Cp). In this study we investigated the presence of specialised transcription factors at all EBNA 2-dependent promoters and how EBNA 2 protein modifications affect its transcription functionality.

### 6.1. The significance of paused RNA Polymerase II at Cp

Initial ChIP assays at Cp proximal regions detected high levels of Pol II, pausing factors DSIF and NELF and the elongation factor pTEFb in association with Brd4. This high level accumulation was not seen at any other latent EBV promoter. Further experiments using nucleosome prediction software (Kaplan et al., 2009) also predicted that Cp and its surrounding sequences had a higher propensity to occlude nucleosomes than other latent promoters (Figure 16A), a feature conserved in both type 1 and type 2 viruses. Confirming the nucleosome prediction, core histone H3 ChIP analysis showed histone occupancy was reduced at Cp. Since NELF has been shown to facilitate Pol II accumulation at promoters that may prevent the formation of nucleosomes, in conjunction with the primary sequence, NELF may also have a role in Cp nucleosome occlusion. NELF knockdown experiments have demonstrated the reduction in paused Pol II at promoters coupled to an increase in histone occupancy (Sun and Li, 2010, Gilchrist et al., 2008). Also, ChIP assays have shown an inverse-promoter occupancy between NELF and core histone H3 (Egloff et al., 2009).

The other pausing factor found at Cp is DSIF. To overcome pausing, Spt5 is phosphorylated by pTEFb (Kim and Sharp, 2001) and unlike NELF, remains bound to the elongating Pol II complex (Zhang et al., 2004), possibly preventing any further downstream pausing events and to promote competent elongation (Cheng and Price, 2007, Zhu et al., 2007). Spt5 can also bind and stimulate the HCE, possibly by stabilising the weak interactions detected between the HCE

and Pol II CTD (Wen and Shatkin, 1999). Previously these pausing factors were thought to prime Pol II at inducible genes such as *Hsp70* to be rapidly induced upon stimulation (Wu et al., 2003a). However, it appears global promoter-proximal pausing occurs at most genes including constitutively active ones in *Drosophila* and mouse embryonic stem cells (Gilchrist et al., 2010, Rahl et al., 2010).

So what is the significance of Cp proximal-pausing? The prevention of Cp-nucleosome formation through the accumulation of paused Pol II may keep Cp in a constitutively open conformation. This could be a reason why EBV switches from Wp to Cp usage during initial infection. Even though Wp and Cp can both transcribe all the EBNA s and immortalise B cells as well as each other at the macroscopic level (Swaminathan, 1996), it would be interesting to compare the kinetics of Pol II elongation from these two promoters. For example; the travelling ratio (TR) of Pol II is the comparison between Pol II occupancy at the 5' end of genes relative to the gene body or 3' end (Reppas et al., 2006). Therefore the closer the TR is to 1 the more efficiently Pol II is retained at promoter-distal levels. We estimate Pol II to have a travelling ratio of 15.77 and 18 at primer sets +30896 to +30994 and +50024 to +50088. This appears quite efficient considering that about 70% of Pol II bound genes in embryonic mouse stem cells have a TR equivalent or higher than this, especially if you consider this Cp value was obtained after Pol II had transcribed 50 kb and the average gene lengths of whole species is considerably less (Rahl et al., 2010). Calculating the TR of Pol II from Wp in Cp deleted or Cp-inactivated mutants could provide direct evidence that Cp transcription is more efficient and justify why there are multiple copies of Wp.

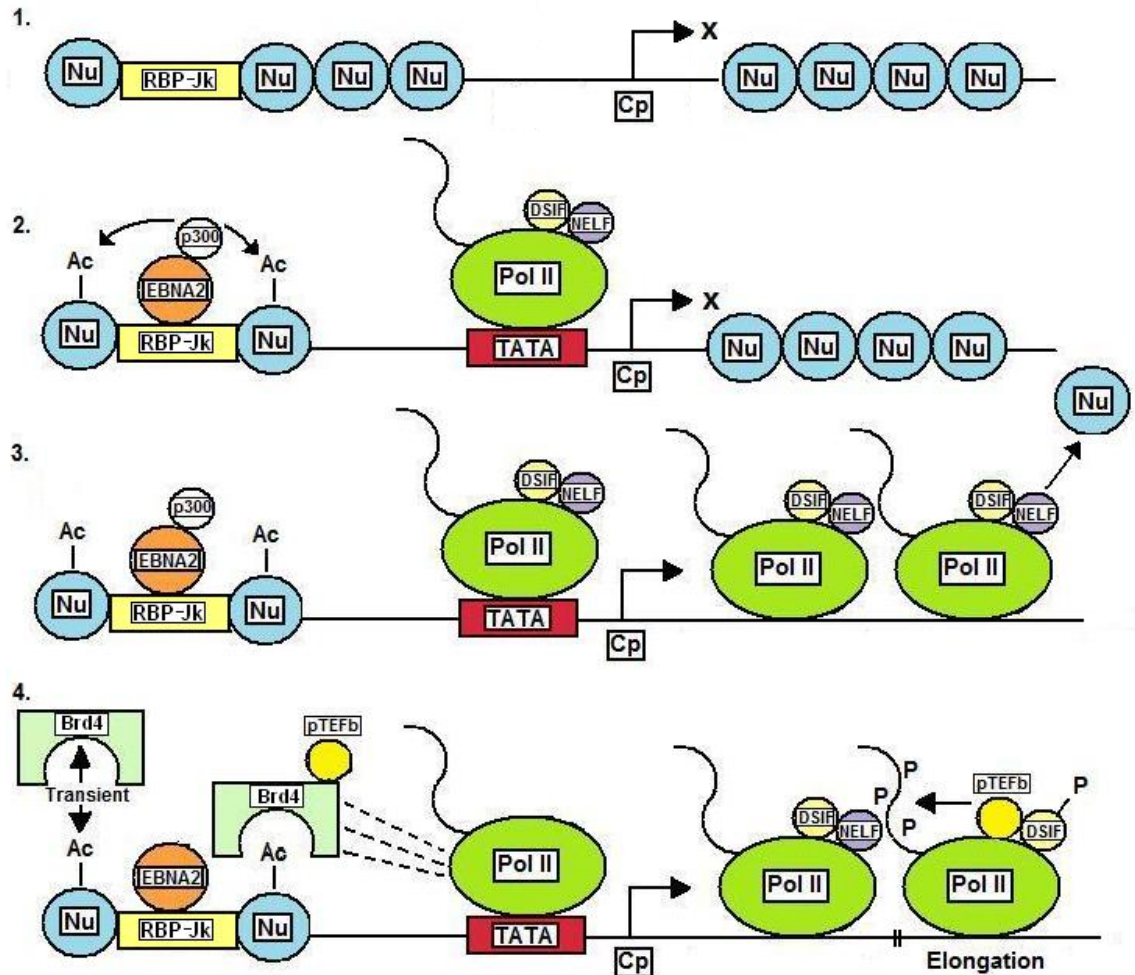
It is widely accepted that pTEFb enhances transcriptional elongation through the phosphorylation of the Pol II CTD, providing a platform for other proteins to bind (For a review see Hirose and Ohkuma, 2007). Since Cp encodes the critically long transcript required for immortalisation, compared to the average human gene length of 15 kb (Strachan and Read, 1999), the absence of specialised mechanisms in which to recruit elongation factors such as pTEFb seems impractical. In failing to detect any interactions between EBNA 2 and pTEFb components, we discovered that pTEFb is recruited to Cp in a Brd4-dependent manner. The interactions between Brd4 and acetylated histones are known to be weak and transient unless

stabilised by other factors (Liu et al., 2008). Thus accumulation of Pol II and transcription factors may provide the scaffold for efficient binding and transfer of pTEFb for elongation. In the presence of the CDK9 inhibitor DRB, Pol II CTD phosphorylation and retention at downstream regions is ablated (Figure 9). Analysing the TR of Pol II in the presence of DRB is dramatically increased to 47.6 and 59 respectively. These data highlight the importance of the specialised recruitment of pTEFb for Cp transcription. Based on our data we propose a model for Cp transcription that is summarised in Figure 46.

## 6.2. JQ1 as a potential PTLD therapy

Brd4 belongs to the highly conserved BET family of proteins which contain two tandem bromodomains (Zeng and Zhou, 2002). The bromodomains associate with acetylated histones transiently unless stabilised by other factors (Liu et al., 2008, Dey et al., 2003). Unlike other BET family members, Brd4 also contains an extra C terminal domain, shown to bind both pTEFb components CDK9 and Cyclin T1 (Bisgrove et al., 2007). Brd4 also associates with viral proteins such as the HPV E2 protein (Wu et al., 2006), KHSV LANA (You et al., 2006) and EBNA 1 (Lin et al., 2008). Since pTEFb is required for efficient Cp transcription, we investigated possible mechanisms of how pTEFb is recruited. Initial experiments demonstrated that neither subunit of pTEFb interacted with EBNA 2 (Figure 22), therefore we investigated the role of Brd4 in pTEFb recruitment to Cp. Using ChIP assays we demonstrated that Brd4 was present at all latency III promoters C, LMP 1 and LMP 2A. Using the bromodomain inhibitor JQ1 (Filippakopoulos et al., 2010), both pTEFb recruitment and Cp transcripts were significantly decreased in the absence of Brd4 (Figures 30 and 31). Therefore both pTEFb recruitment and Cp transcription is dependent on Brd4.

About half of Post-transplant lymphoproliferative disease (PTLD) develops as a result of delayed EBV primary infection (Haque et al., 1996, Ho et al., 1988a). EBV<sup>+</sup> PTLD arises when EBV<sup>+</sup> donors donate blood or organs to EBV sero-negative recipients (Alfieri et al., 1996, Cen et al., 1991, Gerber et al., 1969). The problem is exacerbated by the use of immunosuppressive drugs that primarily prevent organ rejection but also augment EBV infection. Current treatments

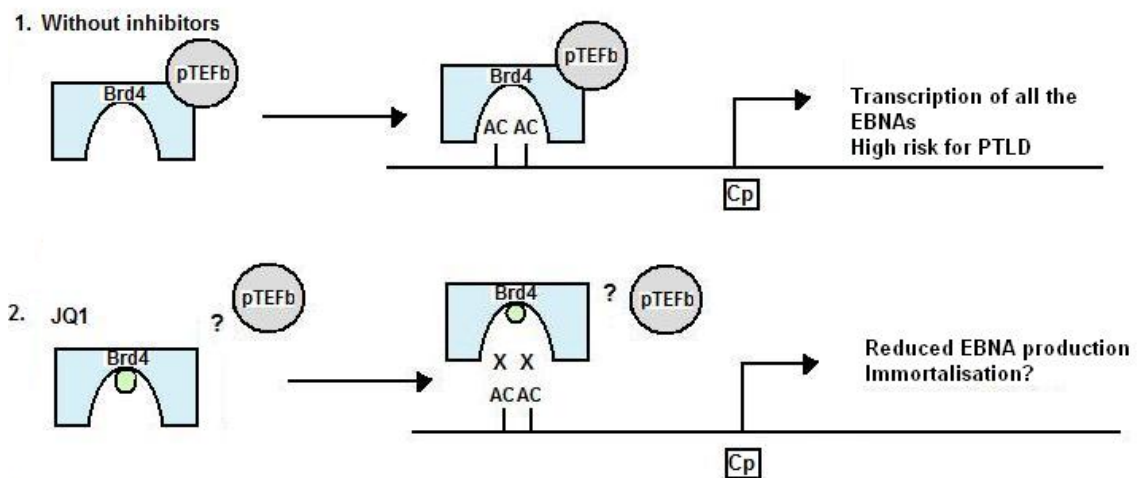


**Figure 46 A model of Cp transcription.** 1. Prior to initial infection and EBNA 2 synthesis, Cp is associated with some nucleosomes (Nu). 2. Upon expression of EBNA 2 from Wp, it binds to the RBP-Jk site located upstream of Cp. Interactions with p300 and SWI/SNF (not shown) chromatin remodellers partially rearrange and acetylate nucleosomes allowing Pol II, the general transcription factors, NELF and DSIF to bind the TATA box. These interactions may be enhanced through EBNA 2. 3. NELF establishes pausing of the transcribing Pol II just downstream of Cp. Nucleosomes are now blocked from re-associating with Cp due to the accumulation of Pol II. This process is enhanced by DSIF, which may facilitate capping and splicing of nascent RNA (not shown). 4. Paused Pol II traverses the TSS enabling stable complexes to be formed with Brd4/pTEFb. pTEFb can now phosphorylate (P) NELF, DSIF and Pol II CTD to promote efficient transcription of all the EBNAs.

for PTLD include the alteration of immunosuppressive drug dosage, anti CD20 (rituximab) and chemotherapy of which none are associated with 100% remission (Dharnidharka and Araya, 2009). Since the JQ1-dependent inhibition of Brd4 resulted in the decreased activity of Cp, we propose JQ1 as a potential treatment for EBV<sup>+</sup> PTLD patients. JQ1 is the first BET family inhibitor to specifically block the association of bromodomains with acetylated histones (Filippakopoulos et al., 2010). In theory, the absence of Brd4 would prevent the specialised recruitment of pTEFb to Cp and compromise transcription of the EBNA<sub>s</sub> and B cell transformation and growth (Figure 47).

Wp is regulated by cellular factors such as PAX5, RTX family members, YY1 and CREB/ATF factors (Tierney et al., 2000a, Kirby et al., 2000, Bell et al., 1998, Tierney et al., 2007). The roles of each transcription factor during Wp transcription are not yet fully defined. Transcription beginning from the last Wp to the EBNA 1 gene is approximately 60kb, substantially larger than the average human gene 10-15kb (including introns) (Strachan and Read, 1999). Therefore Wp-dependent transcription may require Brd4 and specialised elongation factors such as pTEFb and DSIF. Initial ChIP assays targeting Brd4, pTEFb and DSIF in LCLs that contain a Cp deletion such as IB4 or Wp-restricted BL cell lines could elucidate Wp transcription further. Alternatively, CDK9 inhibition assays using DRB or Flavopiridol treatment combined with Wp-transcript quantification may be preferential because our ChIP assay protocol was not sensitive enough to detect pTEFb at Cp distal regions.

Recently, several studies analysing the effects of JQ1 in varying cancer cell lines have implicated the involvement of BET family members in Acute Myeloid Leukaemia (AML) (Mertz et al., 2011, Zuber et al., 2011), BL (Mertz et al., 2011) and Multiple Myeloma (MM) (Delmore et al., 2011). Interestingly, gene expression profiling in the presence or absence of JQ1 revealed the consistent downregulation of c-MYC transcripts in all 3 cancer cell types (Delmore et al., 2011, Mertz et al., 2011). Further ChIP experiments revealed the binding of Brd4 upstream of the c-MYC promoter in AML cells and in the IgH translocated c-MYC locus in MM cells (Delmore et al., 2011, Mertz et al., 2011). In these cancer cell lines it is unclear whether Brd4 is regulating transcription of c-MYC with or without pTEFb recruitment. Transcription of the c-MYC oncogene



**Figure 47 JQ1 as a potential PTLD treatment.** 1 Schematic of EBV infection in an naïve individual. Brd4 in conjunction with pTEFb can bind and activate transcription from Cp. This is potentially a high risk factor for PTLD. 2 Conversely, treatment using JQ1 would prevent the association of Brd4 with acetylated histones. This would prevent the specialised recruitment of pTEFb to Cp and transcription of the EBNAs may be compromised. JQ1-Brd4 may also prevent the association between Brd4 and pTEFb.

is dependent on pTEFb for its constitutive expression (Gargano et al., 2007). Also, HEXIM-1, known to bind pTEFb in the inactive complex, was upregulated upon JQ1 addition in two independent studies (Delmore et al., 2011, Mertz et al., 2011). Therefore JQ1 could act by directly inhibiting the binding of Brd4 to chromatin and act as a non-competitive inhibitor for pTEFb binding. The remaining pTEFb would then be sequestered by the excess HEXIM-1.

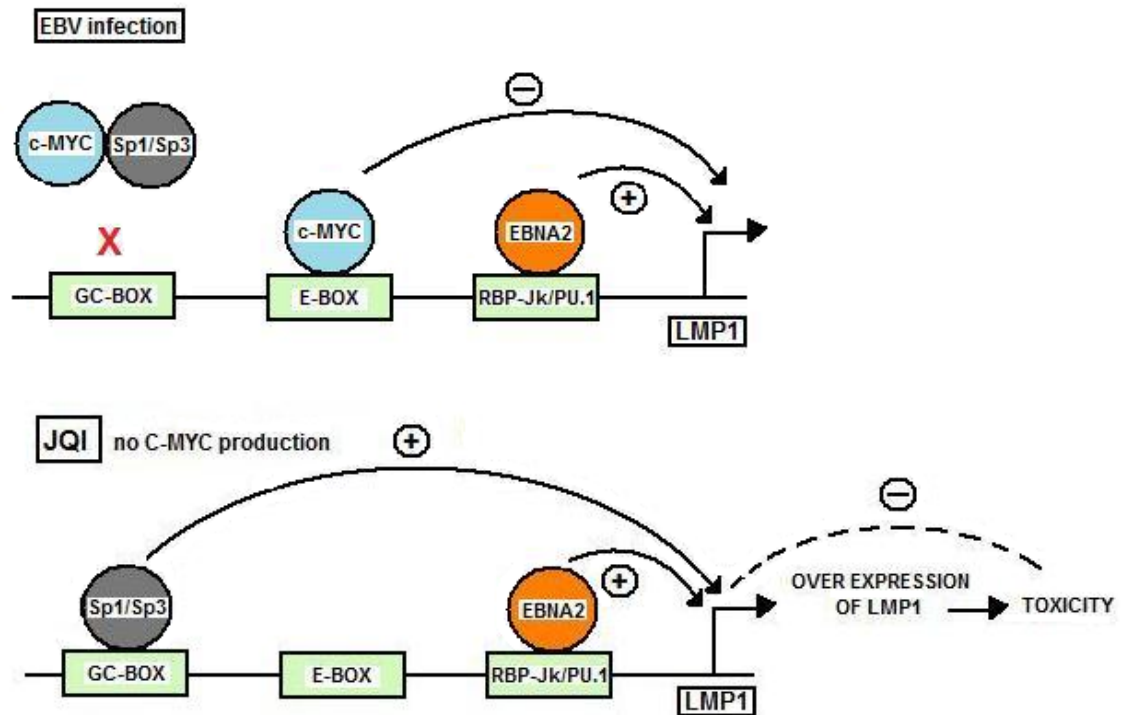
Since our JQ1 experiments were carried out in a BL we cannot rule out the possibility that contributions to Cp and LMP1 transcription are made by c-MYC. Several studies have shown c-MYC is able to recruit pTEFb to promoters to stimulate transcription (Eberhardy and Farnham, 2001, Eberhardy and Farnham, 2002, Kanazawa et al., 2003). However, our Cp data suggests that it is Brd4 that recruits pTEFb to Cp (Figure 27). Whether c-MYC enhances this recruitment is unknown. Cp mRNA studies, pTEFb and c-MYC ChIP in the presence of c-MYC inhibitors could determine a role for c-MYC at Cp. The excess of c-MYC in BL cell lines could make more active pTEFb available for Cp to overcome pausing.

Global transcription factor binding studies in embryonic stem cells demonstrated that c-MYC is present at a third of all active genes and is required to overcome some transcriptional pausing (Rahl et al., 2010). Our results demonstrated that JQ1 treatment in Mutu III cells after 24 hrs led to a 5 fold upregulation of LMP1 transcripts (Figure 30). Concomitantly, after 48 hrs, LMP 1 transcripts were at control levels but associated with the accumulation of Pol II at LMP1p (Figure 32). The LMP1p contains an upstream GC box, found to bind the transcription factors Sp1 and Sp3, which are required for LMP1p transcription (Tsai et al., 1999). Additionally, c-MYC can bind and sequester Sp1 and Sp3 away from chromatin, resulting in transcriptional repression (Gartel et al., 2001). In theory, c-MYC may bind the Sp1/Sp3 transcription factors in a B cell environment to regulate the activation of LMPp1. In the presence of JQ1, an absence of Brd4-dependent c-MYC transcription could permit the association of Sp1/Sp3 and LMPp1 transcription can occur. By 48 hours LMP1 transcription is back to control levels, possibly through a negative feedback loop because LMP1 overexpression is toxic (Figure 47) (Hammerschmidt et al., 1989, Gottschalk et al., 2003, Le Clorennec et al., 2008).

Another potential regulatory mechanism of EBV latent genes by c-MYC is through the direct binding of the c-MYC/MAX heterodimer to their cognate DNA binding sequence, the E-box

(Blackwell et al., 1990, Blackwood and Eisenman, 1991, Halazonetis and Kandil, 1991). Although c-MYC transcriptional repression has not been shown to involve DNA binding (For a review see Gartel, 2006). Observations that EBNA 2 induces c-MYC expression early during B cell infection and that c-MYC plays a crucial role in the proliferation of LCLs does suggest a role for c-MYC in EBV latency (Faumont et al., 2009, Alfieri et al., 1991). ChIP assays targeting c-MYC in both LCLs and BL cell lines would show any association with EBV latent promoters. If c-MYC is present, the use of JQ1 or c-MYC inhibitors followed by the quantification of mRNA expression could be used to clarify c-MYC function. Additionally, LMP1p and Cp reporter constructs (with and without mutations) co-expressed with c-MYC siRNA or with a c-MYC inhibitor could be performed in an LCL.





**Figure 48 The potential c-MYC regulation of LMP1p.** During EBV infection, c-MYC may negatively regulate LMP1p through two independent mechanisms. Firstly, by binding and sequestering transcription factors away from LMP1p that are required for its activation. The binding of c-MYC to the E-Box located upstream of the LMP1p may also have similar effects (top diagram). In the presence of JQ1 and the subsequent suppression of c-MYC expression, it is unable to regulate the LMP1p. Therefore LMP1p is negatively regulated by a feedback loop because the over-expression of LMP1p is toxic (bottom diagram)

### 6.3. The significance of EBNA 2 methylation

EBNA 2-dependent transcription is regulated by phosphorylation and the availability of other viral and cellular factors. EBNA 2 cannot directly bind directly to DNA, instead it interacts with the adapter proteins RBP-Jk and PU.1 through its CR6 domain to facilitate transcription (Zhou et al., 2000a, Ling et al., 1993, Johannsen et al., 1995). These associations are regulated through the hyperphosphorylation of the EBNA 2 serine 243 residue during the cell cycle or lytic cycle reactivation (Yue et al., 2006, Yue et al., 2005, Yue et al., 2004). Subsequent hyperphosphorylation leads to the disassociation of EBNA 2 from DNA. EBNA 1 and EBNA-LP can both enhance EBNA 2-dependent Cp and LMP1p transcription (Sugden and Warren, 1989, Reisman and Sugden, 1986, Gahn and Sugden, 1995, Nitsche et al., 1997, Harada and Kieff, 1997). In addition EBNA 3C can also positively regulate LMPp1 transcription as well as the EBNA 2 target, CD21 (Jimenez-Ramirez et al., 2006, Marshall and Sample, 1995, Wang et al., 1990a).

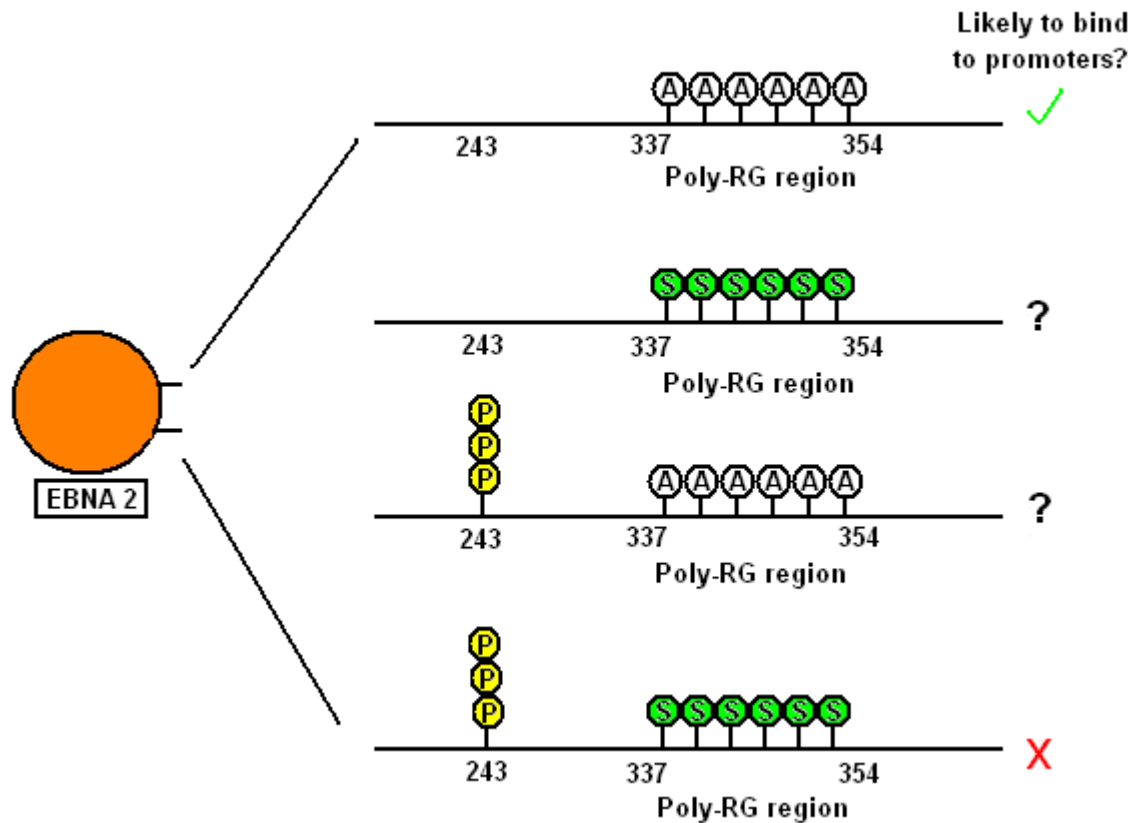
The poly-RG region of EBNA 2 (amino acids 337 to 354) is critical but not essential for B cell transformation (Tong et al., 1994). Interestingly, the deletion of the poly-RG region led to an upregulation of LMP transcripts *in vitro* (Tong et al., 1994). The poly-RG has been shown to be symmetrically di-methylated (sDMA) on its arginine residues permitting the binding of the survival motor neuron protein SMN (Barth et al., 2003, Voss et al., 2001). Arginine methyltransferases (PRMTs) are responsible for the methylation of arginine residues. Type II PRMTs catalyse sDMA modification whereas type I PRMTs catalyse asymmetric dimethylation (aDMA) modification (For a review see Wolf, 2009). In collaboration with the Grässer lab, we investigated the significance of sDMA modification of EBNA 2. Differentially methylated EBNA 2 clearly displayed different DNA binding capacities. aDMA-EBNA 2 bound the viral Cp, LMP1p and LMP2Ap at much higher levels than sDMA-EBNA 2 and cellular CD23 and CD79B promoters. Therefore, in addition to phosphorylation, methylation also regulates EBNA 2-transcriptional regulation.

This differential methylation of EBNA 2 may play a key role during EBV infection. The inability of EBNA 2 to bind and transactivate the latency III growth programme is important

during infection of a healthy host. This permits the alternate, less immunogenic latency types to dominate and establish life long persistence. This down regulation, occurring during the B cell GC reaction, after naïve B cells encounter a pathogen for the first time (Thorley-Lawson, 2001), ensures a safe, permanent passage into the memory B cell pool.

There are currently 11 PRMTs, 5 class I (catalyse aDMA), 2 class 2 (catalyse sDMA) and 4 are unknown (Wolf, 2009). It would be interesting to investigate how the PRMTs responsible for EBNA 2 methylation are expressed/regulated during EBV infection. Isolating the PRMT(s) responsible for modifying EBNA 2 would be important. One way to detect these interactions is through either endogenous or exogenous expression of each PRMT and subsequent immunoprecipitations against methylated forms of EBNA 2 in an EBV latency III background.

Identifying the PRMTs would allow further experiments determining whether the methylation and phosphorylation of EBNA 2 are mutually exclusive or pre-requisites for one another (Figure 49). For example, does the phosphorylation of EBNA 2 first require sDMA methylation of the poly-RG? Or vice versa? Likewise, does aDMA-EBNA 2 prevent the hyperphosphorylation at serine 243? Site-directed mutagenesis of the serine 243 to a glutamic acid residue would mimic phosphorylation at this site. Subsequent *in vivo* binding assays could determine the existence of both sDMA and phosphorylated forms of EBNA 2. Alternatively, *in vitro* kinase assays using CDK1 or BGLF4 and the different methylated forms of EBNA 2 could be used.



**Figure 49 The possible permutations of EBNA 2 in the regulation of its binding to promoters.** EBNA 2 may exist in 4 different permutations, shown here is the likelihood of these different EBNA 2 combinations to bind at promoters. EBNA 2 is most likely to bind DNA in an aDMA form (A) without hyperphosphorylation (top picture). Conversely, EBNA 2 is least likely to bind DNA with both serine 243 hyperphosphorylation (P) and the sDMA modification (S). The middle two permutations represent the unknown, whether sDMA-EBNA 2 in the absence of hyperphosphorylation or aDMA-EBNA 2 with hyperphosphorylation can bind to promoters remains to be determined.

#### 6.4. The role of EBNA 2 in transcriptional repression

The mechanism of EBNA 2-dependent downregulation of cellular genes has not been deduced. Unlike EBNA 3C, EBNA 2 has not been shown to recruit any co-repressive complexes or stimulate negative histone marks associated with transcriptional inhibition. EBNA 2 could either act directly at a promoter or indirectly through the upregulation of repressor proteins. CD79B is a component of the BCR and was downregulated in multiple microarray studies including LCLs, a pair of EBV negative BL cell lines conditionally expressing EBNA 2 and GC B cells (Cahir-Mcfarland et al., 2004, Faumont et al., 2009, Maier et al., 2006, Vockerodt et al., 2008).

The CD79B (B29) promoter contains 2 highly conserved PU.1 sites, one of which binds PU.1 *in vivo* (Thompson et al., 1996, Omori and Wall, 1993). Due to the known interactions between EBNA 2 and PU.1 and their role in LMP1 transcription (Johannsen et al., 1995), we investigated whether EBNA 2 could bind the B29 promoter and regulate CD79B expression. We demonstrated the binding of EBNA 2 to B29 which coincided with the downregulation of CD79B transcripts (Figures 44 and 45). Additionally, EBNA 2 peak binding coincided with both the conserved PU.1 binding sites. To verify EBNA 2 interacts with PU.1 at the CD79B gene, gel shift assays using a wildtype B29 promoter and mutations within the two PU.1 sites could be performed. Conversely wildtype or EBNA 2 mutants unable to bind PU.1 could be transiently co-transfected with a B29 reporter into EBV negative BL cells.

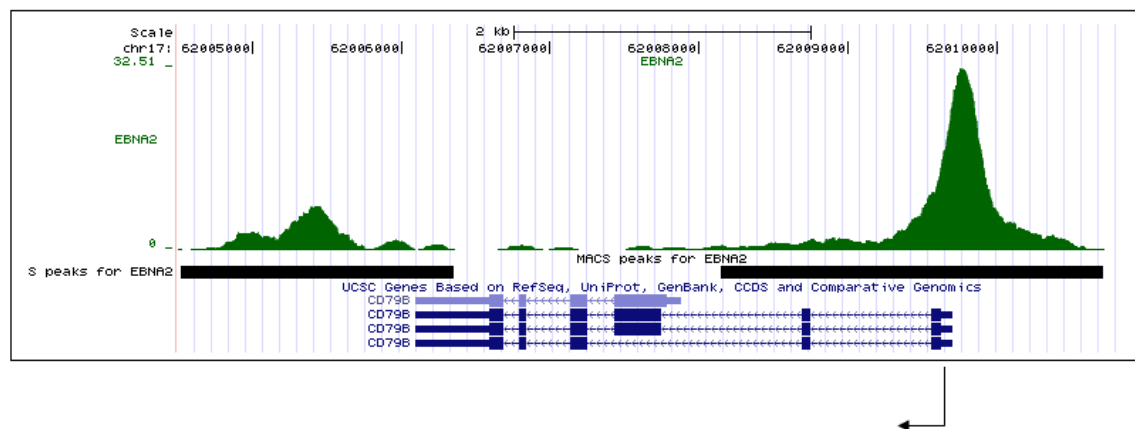
The B29 gene is regulated by the B-cell transcription factor Bob1 and a 3' enhancer element (Malone and Wall, 2002, Malone et al., 2006). Interestingly, the Bob1 binding site is located within 3 base pairs of the known PU.1 binding site (Thompson et al., 1996). Therefore the binding of both EBNA 2 and Bob1 to the B29 gene could be mutually exclusive. ChIP assays using a Bob1 antibody in Mutu I and Mutu III cells or gel shift assays with increasing amounts of EBNA 2 and Bob1 could be used to verify this.

EBNA 2 ChIP coupled to next generation sequencing was performed in Mutu III cells by Michael McClellan from our laboratory. Inspection of the EBNA 2 binding profile at the CD79Bp identified two distinct peaks, both just outside the coding regions at opposite ends of the B29

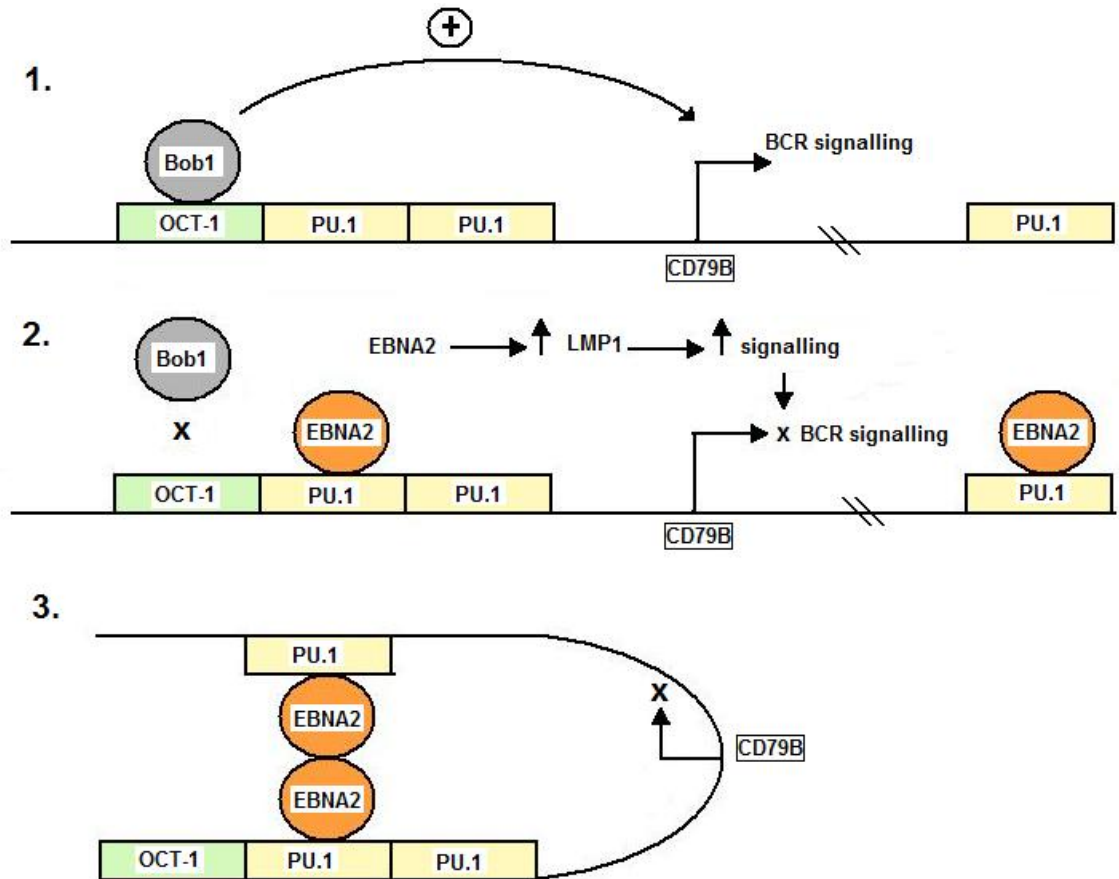
gene (Figure 50). Coupled with the ability of EBNA 2 to self associate (Grasser et al., 1991, Tsui and Schubach, 1994) this could cause a DNA looping effect, as the EBNA 2 at the promoter interacts directly with EBNA 2 found at the 3' end, thus preventing promoter access as shown in Figure 51. The binding of EBNA 2 at the promoter and near the 3' enhancer causes DNA looping, stabilised by the two adjacent EBNA 2 molecules. Initial experiments could be carried out using reporter assays with various plasmids containing mutations in both of the EBNA 2 binding regions that flank the B29 gene. In addition, chromosome conformation capture (3C) techniques could be used to detect any DNA looping. Interestingly, plasmids expressing LMP1 in EBV negative GC B cells also downregulated CD79B expression (Vockerodt et al., 2008). Further experiments have shown this downregulation to be independent of NF- $\kappa$ B signalling (Cahir-Mcfarland et al., 2004).

The roles of EBNA 2 and LMP1 in the downregulation of CD79B are not fully understood. Additional B-cell transcriptional regulatory networks involving Bob1 and ID2, known to inhibit B cell factors important for differentiation, may also be involved (Becker-Herman et al., 2002, Vockerodt et al., 2008). It appears EBV may employ many mechanisms to downregulate BCR signalling, possibly to prevent BCR-dependent activation of the lytic cycle and to remain invisible to the immune system (Miller et al., 1993, Miller et al., 1995).

In this work we have provided evidence for the specialised recruitment of key transcription factors that ensure Cp transcription and EBNA production. Also, we have shown the Cp sequence differs from all other EBV latent promoters. By having a lower propensity to accommodate nucleosome formation, Pol II can occupy the promoter and prevent Cp repression. We also demonstrate the methylation of the poly-RG region of EBNA 2 functionally regulates binding to promoters and has implications for EBV latency switching during infection. Furthermore, understanding how EBV regulates Cp and cellular genes will ultimately help define new, therapeutic targets of use in the development of strategies to treat EBV-associated diseases.



**Figure 50 EBNA 2 binding at the CD79B gene.** ChIP coupled to next generation sequencing carried out in Mutu III cells. Multiple sequence reads from areas of EBNA 2 enriched DNA are plotted with the subtraction of background signal and aligned with the human genome. Two MACs peaks are indicated (black boxes) suggesting there are two EBNA 2 binding sites within CD79B gene locus. One that coincides with the transcriptional start site and one at the 3' end of the gene. The arrow indicates the direction of transcription.



**Figure 51 The possible actions of EBNA 2 and LMP1 in the downregulation of CD79B.** 1. A basic schematic representing the normal transcription of CD79B in uninfected B cells. A global B cell regulator, Bob1, binds upstream and activates transcription of CD79B through the OCT-1 binding motif. Normal BCR signalling permits the differentiation and proliferation of the B cell. 2 During EBV infection, EBNA 2 competes with Bob1 for CD79B promoter proximal binding and can bind at downstream regions. EBNA 2 also upregulates the transcription of the LMP1p causing the increase in signalling pathways. The cumulative effect is the downregulation of CD79B and BCR signalling. 3 EBNA 2 binding at both the 5' and 3' end of CD79B may create a DNA looping effect, stabilised by the interactions of multiple EBNA 2 subunits.



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**Appendix A** Antibodies used in ChIP (normal method)

<b>Protein</b>	<b>Antibody</b>	<b>Antibody species</b>	<b>Quantity used (µg)</b>	<b>Company/ generated by</b>	<b>Secondary antibody</b>	<b>Quantity used (µg)</b>	<b>Company/ generated by</b>	<b>Notes</b>
Brd4	Brd4 (sc-48772)	Rabbit polyclonal	5	Santa Cruz	-	-	-	-
CDK9	CDK9 (sc-8338)	Rabbit polyclonal	5	Santa Cruz	-	-	-	-
Cyclin T1	Cyclin T1 (sc-10750)	Rabbit polyclonal	5	Santa Cruz	-	-	-	-
DSIF	Spt5 (sc-28678)	Rabbit polyclonal	5	Santa Cruz	-	-	-	-
EBNA 2	PE2	Mouse monoclonal	8	M. Rowe	rabbit anti mouse IgG	13.5	Dako	Primary added first
FACT	Spt16 (sc-28734)	Rabbit polyclonal	5	Santa Cruz	-	-	-	-
Histone H3	Total H3 (ab 1791)	Rabbit polyclonal	2	Abcam	-	-	-	-
Histone H3	Di-acetylated Histone H3 (Q16695 )	Rabbit polyclonal	5	Upstate	-	-	-	-
Histone H3	Tri-methylated K27 (17-622)	Rabbit polyclonal	3	Upstate	-	-	-	-
Histone H4	Di-acetylated Histone H4 (06-598)	Rabbit polyclonal	5	Upstate	-	-	-	-
p300	p300 (sc-585)	Rabbit polyclonal	5	Santa Cruz	-	-	-	-
Pol II	Pol II (sc-899)	Rabbit polyclonal	5	Santa Cruz	-	-	-	-
TBP	TBP (sc-204)	Rabbit polyclonal	5	Santa Cruz	-	-	-	-

**Appendix B** Antibodies used in ChIP (pre-absorbed method)

Protein	Antibody	Antibody species	Quantity used (µg)	Company/generated by	Secondary antibody	Quantity used (µg)	Company	Notes
Asymmetric dimethylated EBNA 2	aDMA (6F12)	Mouse Monoclonal	Supernatants	F. Grasser	rabbit anti mouse IgG	13.5	Dako	Secondary Ab added first
EBNA 2	R3	Rat Monoclonal	Supernatants	F. Grasser	-	-	-	-
Non specific Mouse	Mouse IgG	Mouse Monoclonal	Supernatants	F. Grasser	rabbit anti mouse IgG	13.5	Dako	Secondary Ab added first
Non specific Rat	Rat IgG	Rat Monoclonal	Supernatants	F. Grasser	-	-	-	-
NELF	NELF A (sc-23599)	Goat polyclonal	5	Santa Cruz	-	-	-	-
Phosphorylated Ser 2	H5 (MMS-129R)	IgM Mouse monoclonal	25	Covance	rabbit anti mouse IgM	10	Dako	Secondary Ab added first
Phosphorylated Ser 5	H14 (MMS-134R)	IgM Mouse monoclonal	5	Covance	rabbit anti mouse IgM	10	Dako	Secondary Ab added first
Symmetric dimethylated EBNA 2	sDMA (13B10)	Rat Monoclonal	Supernatants	F. Grasser	-	-	-	-
Symmetric dimethylated EBNA 2	sDMA (7D9)	Mouse Monoclonal	Supernatants	F. Grasser	rabbit anti mouse IgG	13.5	Dako	Secondary Ab added first

**Appendix C** Antibodies used in Western blotting

<b>Protein</b>	<b>Antibody</b>	<b>Antibody species</b>	<b>Antibody dilution</b>	<b>Company/ generated by</b>	<b>Secondary antibody</b>	<b>Antibody dilution</b>	<b>Company/ generated by</b>
Actin	Actin (A2103)	Rabbit polyclonal	1 in 5000	Sigma	Anti Rabbit-HRP	1 in 5000	Cell Signalling
Brd4	Brd4 (A301-985A)	Rabbit polyclonal	1 in 1000	Bethyl Labs	Anti Rabbit-HRP	1 in 2000	Cell Signalling
CDK7	CDK7 (sc-529)	Rabbit polyclonal	1 in 500	Santa Cruz	Anti Rabbit-HRP	1 in 3000	Cell Signalling
CDK8	CDK8 (sc-5612)	Goat polyclonal	1 in 1000	Santa Cruz	Rabbit anti Goat-HRP	1 in 1000	Dako
CDK9	CDK9 (sc-7331)	Mouse monoclonal	1 in 1000	Santa Cruz	Anti Mouse-HRP	1 in 5000	Cell Signalling
Cyclin T1	Cyclin T1 (sc-10750)	Rabbit polyclonal	1 in 1000	Santa Cruz	Anti Rabbit-HRP	1 in 5000	Cell Signalling
EBNA 1	M.S. Serum	Human	1 in 200	M. Rowe	Protein A HRP	1 in 1000	Dako
EBNA 2	PE2	Mouse monoclonal	1 in 1000	M. Rowe	Rabbit anti Mouse-HRP	1 in 1000	Dako
LMP 1	CS1-4	Mouse monoclonal	1 in 300	M. Rowe	Anti Mouse-HRP	1 in 3000	Cell Signalling
NELF	NELF-A (sc-23599)	Goat polyclonal	1 in 300	Santa Cruz	Rabbit anti Goat-HRP	1 in 1000	Dako

### Appendix D Real-time PCR primers used in ChIP assays

Name	Location <sup>a</sup>	B95-8 sequence number <sup>b</sup>	Sequence (5' to 3')
<i>Cp primer sets</i>			
Forward (MW 428)	-1409 to -1392	9926 to 9943	GGAGGAAGTCATGCCACA
Reverse (MW 429)	-1310 to -1293	10025 to 10042	AGGAACCTGTGGCACCTG
Forward (MW 70)	-430 to -410	10905 to 10925	CCTAGGCCAGCCAGAGATAAT
Reverse (MW 71)	-356 to -337	10979 to 10998	AGATAGCACTCGACGCACTG
Forward (MW 133)	-208 to -189	11127 to 11146	ACCTTAGAGGTGGAGCAACG
Reverse (MW 134)	-114 to -96	11221 to 11240	GGCGAATTAAGTGGAGCTTGC
Forward (MW 432)	-107 to -89	11228 to 11246	CAGTTAATTCGCCCACGAC
Reverse (MW 433)	-23 to -2	11312 to 11333	AATTTGCAGCAGAACACAGAGT
Forward (MW 155)	+48 to +67	11383 to 11402	CATCGCAGGGTTCTTACCAT
Reverse (MW 156)	+148 to +167	11483 to 11502	CCTCAGGAGGCCCTTAGACT
Forward (MW 157)	+295 to +313	11630 to 11649	GAAGAAACAGCCTCCTGCAC
Reverse (MW 158)	+387 to +406	11722 to 11741	TTCAGTGCCCAGATTCATGT
Forward (MW 274)	+2590 to +2605 (first repeat) <sup>c</sup>	13925 to 13940	CCAGGCCTGCCAAAGA
Reverse (MW 275)	+2636 to +2654 <sup>c</sup>	13971 to 13989	CTGTTACTGCACCCGCTTT
Forward (MW 226)	+30896 to +30916 <sup>d</sup>	42231 to 42251	GCGTTATCATGTGTTGCTTGA
Reverse (MW 227)	+30973 to +30994 <sup>d</sup>	42308 to 42329	AGATCCACATGTTGCGGTGTG
Forward (MW 280)	+38667 to +38686	50002 to 50021	CTGTCACCACCTCCCTGATA
Reverse (MW 281)	+38712 to +38731	50047 to 50066	GAACACTCCCTCAGTGGTCA

Name	Location <sup>a</sup>	B95-8 sequence number <sup>b</sup>	Sequence (5' to 3')
<b>Forward (MW 282)</b>	+38828 to +38847	50163 to 50182	GATGGCGGGTAATACATGCT
<b>Reverse (MW 283)</b>	+38903 to +38922	50238 to 50257	CAAACATACACCGTGCGAAA
<b>Forward (MW 288)</b>	+40493 to +40512	51828 to 51847	TCGAAAGACTCGGAGTAGCC
<b>Reverse (MW 289)</b>	+40542 to +40557	51878 to 51892	TCCCTGGTGGAGACC
<b>Forward (MW 290)</b>	+50024 to +50044	61359 to 61379	GACCCATAGCAGAGACCTGAG
<b>Reverse (MW 291)</b>	+50071 to +50088	61407 to 61423	CGGCCGAGTATGAGCAG
<b>Forward (MW 161)</b>	+59014 to +59033	70349 to 70368	TGAGGCATCCGATATGACTG
<b>Reverse (MW 162)</b>	+59083 to +59104	70418 to 70439	CTCCACCTGTGTAACCTCACTG
<b><i>LMP2Ap / (LMP1p) primer sets<sup>e</sup></i></b>			
<b>Forward (MW 357)</b>	-268 to -246	165771 to 165793	GATAGCCTCGCGACTCGTGGGAA
<b>Reverse (MW 358)</b>	-205 to -185	165834 to 165854	AATCTTCACACACTGCTGCTG
<b>Forward (MW 359)</b>	+150 to +170	166189 to 166209	CCAATATCCATCTGCTTCTGG
<b>Reverse (MW 360)</b>	+208 to +231	166247 to 166270	GGCTCTTCATTAGATTCACGTTG
<b>Forward (MW 361)</b>	+381 to +406 (+2637 to +2611) <sup>f</sup>	166420 to 166446	CTCATCTCAACACATATATGAAGAAGC
<b>Reverse (MW 362)</b>	+451 to +472 (+2567 to +2546)	166490 to 166511	TTGATGTGACTTGTGATGCAAT
<b>Forward (MW 144)</b>	+695 to +712 (+2323 to +2306)	166734 to 166751	GGACACGCTCCTTCTTGG
<b>Reverse (MW 143)</b>	+759 to +780 (+2259 to +2238)	166798 to 166819	ACTGGCTGGATTCTACGCTACT
<b>Forward (MW 150)</b>	+1346 to +1367 (+1672 to +1651)	167385 to 167406	GTGTTGTGCAGAGGTCTGATG
<b>Reverse (MW 149)</b>	+1435 to +1454 (+1583 to +1564)	167474 to 167493	CCTTCACACACCACACAGGT
<b>Forward (MW 148)</b>	+2361 to +2381 (+657 to +637)	168400 to 168420	TGAGCAGGATGAGGTCTAGGA
<b>Reverse (MW 147)</b>	+2424 to +2444 (+594 to +574)	168463 to 168483	GGAGATTCTCTGGCGACTTG



Name	Location <sup>a</sup>	B95-8 sequence number <sup>b</sup>	Sequence (5' to 3')
<b>Forward (MW 140)</b>	+2878 to +2898 (+140 to +120)	168917 to 168937	AGAGGAGGAGAAGGAGAGCAA
<b>Reverse (MW 139)</b>	+2966 to +2984 (+52 to +34)	169005 to 169023	CCTGAGGATGGAACACGAC
<b>Forward (MW 311)</b>	+3249 to +3266 (-231 to -248)	169288 to 169305	GGCCAAGTGCAACAGGAA
<b>Reverse (MW 310)</b>	+3315 to +3335 (-297 to -317)	169354 to 169374	GCAGATTACACTGCCGCTTC
<b><i>OriP primer set</i></b>			
<b>Forward (MW 617)</b>		7036 to 7055	GGAGGAGAAGAGAGGCTTCC
<b>Reverse (MW 618)</b>		7106 to 7125	AATAGCGGACAAGCCGAATA
<b><i>CD23p primer sets</i></b>			
<b>Forward (MW 532)</b>	-471 to -451		TCCTCTGCCTCTCTCTCCTCT
<b>Reverse (MW 533)</b>	-396 to -372		GATTATGTCTCTTGATGATTGAGCA
<b>Forward (MW 534)</b>	-198 to -179		CGCTACCACTCACCTCCTTC
<b>Reverse (MW 535)</b>	-106 to -87		CGGGTGAAGTCCGTGTTATT
<b>Forward (MW 536)</b>	+414 to +433		GACTCTAGGCAGAGGCAGGA
<b>Reverse (MW 537)</b>	+113 to +533		TTGCTCAATCATCTGGAGACA
<b><i>CD79Bp primer sets<sup>g</sup></i></b>			
<b>Forward (MW 574)</b>	-643 to -624		CCTCTCACCAAGACCTGGAC
<b>Reverse (MW 575)</b>	-582 to -563		GAGCCACAGAGGTTCTGGAG
<b>Forward (MW 576)</b>	-460 to -440		TGTCCTTCTTCAGTGCTCCTC
<b>Reverse (MW 577)</b>	-392 to -372		GGCACCTGTCCTATGAATGAA
<b>Forward (MW 578)</b>	+38 to +55		GGACAGAGCGGTGACCAT

Name	Location <sup>a</sup>	B95-8 sequence number <sup>b</sup>	Sequence (5' to 3')
Reverse (MW 579)	+108 to +129		GTTCTGTACCTGAGAGCAGCAG
<i>GAPDH primer set</i>			
Forward (MW 208)	+1948 to +1968		CTCATGCCTTCTTGCCTCTT
Reverse (MW 209)	+2027 to +2048		TTGATGGCAACAATATCCACTT

### Appendix D Real time PCR primers used in ChIP assays

<sup>a</sup> Primer locations are given relative to the start of the relevant mRNA sequence in the annotated EBV sequence (NC\_007605.1) or the cellular genes CD23, CD79B and GAPDH gene.

<sup>b</sup> Numbers relate to the annotated EBV sequence (NC007605.1)

<sup>c</sup> Primers are part of the Wp repeat region containing approximately 7 repeats of an estimated 3072bp sequence

<sup>d</sup> Sequences obtained from Prof. Paul Lieberman, The Wistar Institute, Philadelphia, USA.

<sup>e</sup> Primer locations for the LMP1 gene located in the reverse orientation are given in parentheses

<sup>f</sup> The LMP1 polyA is located at 166483 to 166488 so MW 361 is outside of the transcription unit

<sup>g</sup> Primer locations were defined in accordance with the major transcriptional start site (Thompson et al., 1996)

### Appendix E Real time PCR primers used in cDNA analysis

Gene/promoter	Sequence (5' to 3')
<b><i>Actin</i></b> Forward (MW 418) Reverse (MW 419)	CTGGCACCACACCTTCTACA TTGCTATCCAGGCTGTGCTA
<b><i>CD23</i></b> Forward (MW 572) Reverse (MW 573)	TTCGGAACCTTGGACCTGAAG GCATCATCACGCAGTCCTC
<b><i>CD79B</i></b> Forward (MW 584) Reverse (MW 585)	AGCAGAGGAACACGCTGAA AGCCTTGCTGTCATCCTTGT
<b><i>Cp*</i></b> Forward (MW 302) Reverse (MW 303)	GATCAGATGGCATAGAGACAAGGAC AGGCTGTTTCTTCAGTC
<b><i>NELF 1</i></b> Forward (MW 528) Reverse (MW 529)	ACATCCGTCTCTGCTTCCAT GCCCTTCATCTCGTCCAC
<b><i>NELF 2</i></b> Forward (MW 530) Reverse (MW 531)	GACACAGGCTCGCTTAACCT ATGGCAGACGCTTCACACT
<b><i>Qp*</i></b> Forward (MW 304) Reverse (MW 305)	GGATAGCGTGCGCTACCGGAT CCTCCTCGTCCTCGTCCTCTT

\* Primers were obtained from (Bakos et al., 2007)

# Regulation of transcription by the Epstein–Barr virus nuclear antigen EBNA 2

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## Abstract

The EBNA 2 (Epstein–Barr nuclear antigen 2) transcription factor is essential for B-cell transformation by the cancer-associated EBV (Epstein–Barr virus) and for the continuous proliferation of infected cells. EBNA 2 activates transcription from the viral Cp (C promoter) during infection to generate the 120 kb transcript that encodes all nuclear antigens required for immortalization by EBV. EBNA 2 contains an acidic activation domain and can interact with a number of general transcription factors and co-activators. It is now becoming clear, however, that the regulation of transcription elongation in addition to initiation by EBNA 2, at least in part through CDK9 (cyclin-dependent kinase 9)-dependent phosphorylation of the RNA polymerase C-terminal domain, is likely to play a crucial role in the mechanism of action of this key viral protein.

## Introduction

Greater than 90% of the world's population carry EBV (Epstein–Barr virus) as a persistent asymptomatic infection usually established during childhood. Delayed primary infection can give rise to the benign lymphoproliferation, infectious mononucleosis. Despite its ubiquitous and generally harmless nature, EBV was first isolated from a Burkitt's lymphoma biopsy and has since been causally associated with numerous other cancers including nasopharyngeal carcinoma, Hodgkin's disease and PTL (post-transplant lymphoproliferative disease). EBV has potent transforming activity *in vitro* and is able to infect resting B-cells and drive their uncontrolled growth. The virus establishes a latent infection in these immortalized B-cells and expresses only 11 viral genes, including six EBNAs (Epstein–Barr nuclear antigens; EBNA1, 2, 3A, 3B, 3C and LP) and three LMPs (latent membrane proteins; LMP1, 2A and 2B). All of the EBNAs are able to function as regulators of transcription and EBNA1, 2, 3A and 3C have been shown to be essential for the infection and immortalization process [1–4].

## EBNA 2

EBNA 2 activates cellular and viral transcription through interactions with the cellular DNA-binding proteins RBP-J $\kappa$  (recombination signal-binding protein 1 for J $\kappa$ ) and PU.1 that bind consensus sites in its target promoters [5–9]. EBNA 2 activates the viral Cp (C promoter), from where the long

(up to 120 kb) primary transcript encoding all six of the EBNAs initiates, in addition to the promoters of all three LMP genes [10,11]. EBNA 2 contains an acidic activation domain [12,13] and interacts with a number of general transcription factors and co-activators including TFIIB (transcription factor IIB), TAF40 [TBP (TATA-box-binding protein)-associated factor 40], the p62 and XPD (xeroderma pigmentosum group D) (p80) subunits of TFIIH, a 100 kDa protein that associates with TFIIE, hSNF5, p300, CBP [CREB (cAMP-response-element-binding protein)-binding protein] and PCAF (p300/CREB-binding protein-associated factor) [14–18]. Consistent with the ability of EBNA 2 to recruit HATs (histone acetyltransferases) to promoters, differential histone H3 and H4 acetylation has been detected in the presence of EBNA 2 at target promoters *in vivo* [19]. The transcriptional function of EBNA 2 appears to be inhibited by the phosphorylation of EBNA 2 by CDK1 (cyclin-dependent kinase 1) at Ser-243 during mitosis [20], potentially as a result of reduced binding to one of its cellular DNA-binding partners, PU.1. Hyperphosphorylated EBNA 2 fails to activate the LMP 1 and Cps efficiently [20,21].

## CTD (C-terminal domain) phosphorylation

Although polymerase recruitment and the generation of an 'open' chromatin state are crucial regulatory points in the control of transcription initiation, phosphorylation of the CTD of the largest subunit of pol II (RNA polymerase II) also plays a vital role in the regulation of transcription. The CTD in humans consists of 52 repeats of a heptapeptide sequence and becomes phosphorylated during transcription primarily on Ser-2 and Ser-5 in the sequence. Phosphorylation of the CTD is required for efficient initiation, promoter clearance, elongation and RNA processing, and can be regulated in a gene-specific and activator-specific manner (for a review, see [22]). A number of viral and cellular transcription factors have been shown to regulate CTD phosphorylation by recruiting

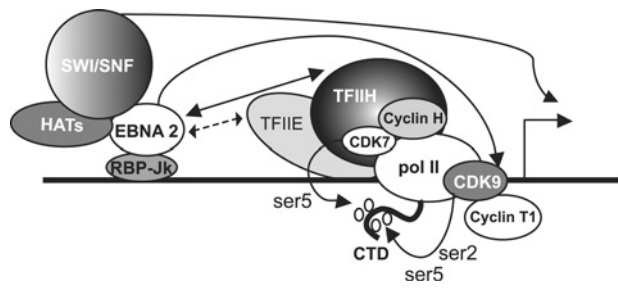
**Key words:** C-terminal domain phosphorylation (CTD phosphorylation), cyclin-dependent kinase 9 (CDK9), elongation, Epstein–Barr nuclear antigen 2 (EBNA 2), Epstein–Barr virus (EBV), transcription.

**Abbreviations used:** CREB, cAMP-response-element-binding protein; Cp, C promoter; CTD, C-terminal domain; DRB, 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole; EBNA, Epstein–Barr nuclear antigen; EBV, Epstein–Barr virus; HAT, histone acetyltransferase; LCL, lymphoblastoid cell line; LMP, latent membrane protein; NELF, negative elongation factor; pol II, RNA polymerase II; pTEF-b, positive transcription elongation factor-b; PTL, post-transplant lymphoproliferative disease; RBP-J $\kappa$ , recombination signal-binding protein 1 for J $\kappa$ ; TFIIB, transcription factor IIB; Wp, W promoter.

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### Figure 1 | A model for the mechanism of transcriptional activation by EBNA 2

EBNA 2 binds HATs and chromatin remodellers (SWI/SNF) to disrupt chromatin structure at the promoter. It also promotes transcription complex assembly by interacting with general transcription factors, e.g. TFIIE and TFIIH. In addition, EBNA 2 promotes transcription initiation and elongation through CDK9-dependent phosphorylation of the CTD on Ser-5 and possibly also Ser-2. TFIIH will also phosphorylate Ser-5 of the CTD during initiation, but there is no evidence that EBNA 2 targets CDK7 to promoters.



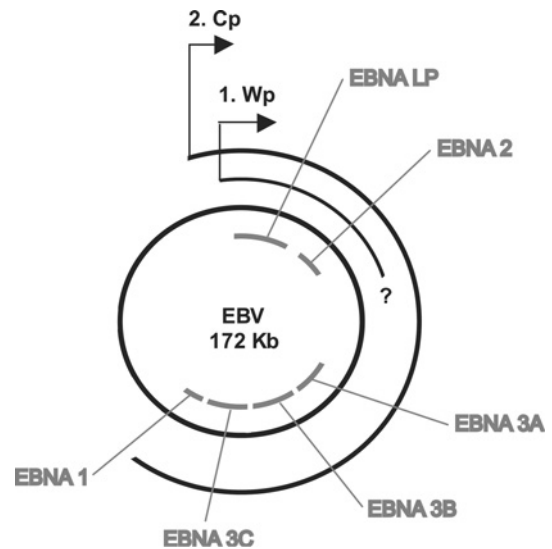
or modulating the activity of the transcription-associated cyclin-dependent CTD kinases, CDK7, CDK8 and CDK9. These include HIV-1 Tat, adenovirus E1A, herpes simplex virus VP16 (viral protein 16), Myc, CIITA (class II transactivator), the androgen receptor and the aryl hydrocarbon receptor [23–28]. CDK7 and cyclin H are subunits of the general transcription factor TFIIH; CDK8 and cyclin C are components of Mediator; CDK9 and cyclin T1 form the pTEF-b (positive transcription elongation factor-b).

### EBNA 2 and CTD phosphorylation

Studies carried out in our laboratory have shown that efficient activation of transcription by EBNA 2 requires the activity of CDK9 [29]. Thus inhibition of CDK9 using a dominant-negative mutant or the CDK9 inhibitor, DRB (5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole), dramatically reduces EBNA 2-activated transcription from the Cps and LMP1 promoters, but has little effect on basal transcription. In addition, using a cell line expressing a conditionally active EBNA 2–oestrogen receptor fusion protein, we were able to detect EBNA 2-dependent increases in the levels of phospho-Ser-5 on the CTD. Although Ser-2 phosphorylation has been closely linked to CDK9 activity, CDK9 phosphorylates Ser-5 and Ser-2 in HIV-1 transcription elongation complexes [30] and has increased activity towards Ser-5 in the presence of HIV-1 Tat [31]. CDK9 has also been shown to have some preference for Ser-5 in peptide studies [32]. These results therefore indicate that the EBNA 2 activation mechanism also involves the potential recruitment of CDK9 to the promoter, leading to increased phosphorylation of Ser-5 and potentially Ser-2 of the CTD (Figure 1). Since CDK9-dependent CTD phosphorylation has been shown to stimulate transcriptional elongation in the HIV-1 Tat transcriptional activation mechanism [27], it is possible

### Figure 2 | A model for the role of EBNA 2 in driving promoter switching during infection

(1) Upon initial infection, poorly processive transcription from the Wp gives rise to some transcripts encoding EBNA LP and EBNA 2. (2) EBNA 2 activates an alternative EBNA promoter (Cp) upstream from Wp and promotes CDK9-dependent processive transcription, leading to the production of full-length mRNAs encoding the remaining EBNAs required for immortalization.



that EBNA 2 may activate transcriptional elongation in addition to initiation through CDK9. This is of particular significance if we consider the role played by EBNA 2 in establishing viral latency and driving the proliferation and immortalization of the infected cell. EBNA 2 is one of the first viral proteins to be expressed after infection (8–12 h) [33,34] with the mRNA encoding EBNA 2 initiating from the Wp (W promoter) under the control of cellular and B-cell-specific transcription factors [35]. Switching of promoter usage from Wp to the upstream Cp begins to occur approx. 48 h post-infection and appears to be a direct consequence of the activation of Cp by EBNA 2 via RBP-J $\kappa$  binding to consensus sites in the promoter regulatory region (Figure 2). Cp transcription results in the expression of the remaining nuclear antigens required for immortalization and is therefore a crucial step in the virus life cycle and the transformation process [36] (Figure 2). We speculate that the driving force behind this switch may be the need to promote processive transcription given that transcription from W leads to the expression of only the most promoter-proximal genes (EBNA 2 and LP) and may thus be predominantly abortive (Figure 2). EBNA 2 activation of the Cp may therefore promote the assembly of elongation-competent transcription complexes capable of efficiently transcribing full-length mRNAs. Interestingly, the Wp is located in the IR-1 (internal repeat 1) region of the EBV genome and is therefore present in multiple copies. This duplication may act to increase the number of initiating polymerases to ensure that sufficient transcripts encoding EBNA 2 are generated early in infection,

since most of the transcription complexes may prematurely disengage from the template. This would, however, represent a very wasteful transcription system and a switch to a more efficient promoter would clearly be beneficial.

## EBNA 2 as a drug target

The dependence of EBNA 2-activated transcription on CDK9 raises the possibility that CDK9-specific CDK inhibitors that have been investigated and found to be wide-spectrum anticancer drugs, such as flavopiridol, could be used as anti-EBV agents. Flavopiridol effectively inhibits CDKs at least 20-fold more selectively than other cellular kinases [37,38] and has been shown to block cell-cycle progression and promote apoptosis through a number of mechanisms, including the inhibition of cell-cycle CDKs, and the inhibition of transcription of anti-apoptotic proteins [39,40]. Significantly, flavopiridol displays most selectivity for CDK9 ( $IC_{50} = 3$  nM) [41]. Given the requirement for CDK9 in the HIV-1 Tat transactivation mechanism [42,43], the potential use of flavopiridol as an anti-HIV agent has been suggested, and studies in mice reported a reduction in HIV-induced disease symptoms in the presence of the drug [44]. Our results identifying a requirement for CDK9 in the EBNA 2 activation mechanism pinpoint a stage in the latent infection process that could be targeted by drugs such as flavopiridol to inhibit EBV-induced B-cell proliferation and immortalization. Potential applications of CDK9-specific drugs may be in the treatment of EBNA 2-positive EBV-associated tumours, such as PTLD, or to block EBNA 2-dependent B-cell proliferation in severe cases of infectious mononucleosis where the use of traditional antivirals is ineffective.

PTLD arises as a direct result of the uncontrolled proliferation of EBV-infected B-lymphocytes, a process that is normally restricted in the presence of an effective T-cell response in healthy EBV-infected individuals. Thus organ or stem cell transplant patients undergoing immunosuppressive therapy are at risk for PTLD development, as are individuals with congenital or acquired immunodeficiencies, e.g. the Wiskott–Aldrich syndrome, ataxia telangiectasia, acute lymphoblastic leukaemia and AIDS. In a large study of solid organ transplant patients, PTLD was found to develop at a frequency of between 1.3 and 8.2%, depending on the organ type, and has been reported to occur in 30% of children undergoing small intestine transplantation [45]. Mortality rates from PTLD have been reported to be as high as 80% for bone-marrow transplant patients [45].

The tumour cells in PTLD resemble the permanently proliferating LCLs (lymphoblastoid cell lines) generated *in vitro* after latent infection of resting B-cells by EBV and therefore express all latent proteins including EBNA 2. EBNA 2 is required for the continued proliferation of EBV-infected cells, and can provide protection from Nur77-mediated apoptosis [46–49]. Agents that specifically target EBNA 2 function would therefore be expected to block not only the initial infection and cellular transformation by EBV, but also the growth of EBV immortalized cell lines and tumour cells that

proliferate in an EBNA 2-dependent manner. Indeed, peptide inhibitors that block the EBNA 2–RBP-J $\kappa$  interaction and prevent transcriptional activation by EBNA 2 have been successfully used to down-regulate EBNA 2 target gene expression, inhibit the growth of LCLs and block EBV transformation of B-cells *in vitro* [49]. First-line treatment for PTLD involves reducing immunosuppression, but this approach runs the risk of inducing transplant rejection. The use of anti-CD20 (B-cell) antibodies has shown reasonable success in phase II trials, but 5-year survival rates still remain unacceptably low [45]. The investigation of new EBV-specific treatment strategies could therefore still be of enormous benefit.

## Future directions

Given the ability of EBNA 2 to promote CTD phosphorylation on Ser-5, it will be interesting to examine the effects of EBNA 2 on the phosphorylation of Ser-2, the main CDK9 CTD target during elongation. It would be likely that the most promoter-distal effects of EBNA 2 would be manifested through phosphorylation of this residue. Moreover, given that CDK9-dependent phosphorylation of the Spt5 subunit of the pausing and elongation regulator DSIF (DRB-sensitivity-inducing factor) plays a role in relieving promoter-proximal pausing induced by NELF (negative elongation factor) and preventing termination, it will be interesting to determine whether these factors are also involved in the EBNA 2 activation mechanism.

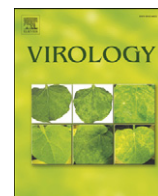
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Received 27 March 2008  
doi:10.1042/BST0360625





## Asymmetric Arginine dimethylation of Epstein–Barr virus nuclear antigen 2 promotes DNA targeting

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### ARTICLE INFO

#### Article history:

Received 13 August 2009

Returned to author for revision

1 September 2009

Accepted 10 November 2009

Available online 6 December 2009

#### Keywords:

Epstein–Barr virus

EBV

Nuclear antigen 2

EBNA2

Methylation

Arginine

sDMA

aDMA

RBPJ $\kappa$

DNA-binding

### ABSTRACT

The Epstein–Barr virus (EBV) growth-transforms B-lymphocytes. The virus-encoded nuclear antigen 2 (EBNA2) is essential for transformation and activates gene expression by association with DNA-bound transcription factors such as RBPJ $\kappa$  (CSL/CBF1). We have previously shown that EBNA2 contains symmetrically dimethylated Arginine (sDMA) residues. Deletion of the RG-repeat results in a reduced ability of the virus to immortalise B-cells. We now show that the RG repeat also contains asymmetrically dimethylated Arginines (aDMA) but neither non-methylated (NMA) Arginines nor citrulline residues. We demonstrate that only aDMA-containing EBNA2 is found in a complex with DNA-bound RBPJ $\kappa$  *in vitro* and preferentially associates with the EBNA2-responsive EBV C, LMP1 and LMP2A promoters *in vivo*. Inhibition of methylation in EBV-infected cells results in reduced expression of the EBNA2-regulated viral gene LMP1, providing additional evidence that methylation is a prerequisite for DNA-binding by EBNA2 via association with the transcription factor RBPJ $\kappa$ .

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### Introduction

The Epstein–Barr virus (EBV) is associated with various human malignancies and readily growth-transforms primary human B-lymphocytes generating lymphoblastoid cell-lines, the *in vitro* correlate of the tumour cells of EBV-associated post-transplant lymphoproliferative disease (PTLD) (for review, see Rickinson and Kieff, 2007). In EBV-transformed lymphocytes, 11 so-called latent genes are expressed. Of these, only the nuclear antigens EBNA-1, -2, -3a, -3c and the latent membrane protein LMP1 are necessary for transformation (reviewed in Bornkamm and Hammerschmidt, 2001).

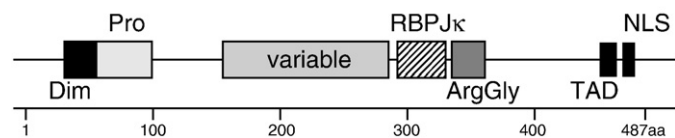
EBNA2 is a multifunctional transcriptional activator (for a recent review, see Palermo et al., 2008). Although it self-associates (Harada et al., 2001), a property often observed for DNA-bound transcription factors, it does not bind directly to DNA but is tethered to promoter elements by interacting with DNA-bound cellular transcription factors. For example, it associates through its Trp-Trp-Pro (“WWP<sub>325</sub>”) motif at positions 323–325 with the DNA-bound repressor RBPJ $\kappa$  (Henkel et al., 1994; Ling and Hayward, 1995; Zimmer Strobl et al., 1993) thereby converting RBPJ $\kappa$  to the transcriptionally active form in an analogous fashion to the cellular transmembrane receptor, Notch (reviewed in Zimmer Strobl and Strobl, 2001). A virus encoding an EBNA2 protein with a mutation in the WWP-motif is unable to immortalise B-lymphocytes and does not activate the viral oncogene LMP1 (Cohen, Wang, and Kieff, 1991). EBNA2 binds to a variety of basal transcription factors (Bornkamm and Hammerschmidt, 2001) and also forms complexes with proteins involved in RNA metabolism like the DEAD-box protein DDX20 (DP103/Gemin3) (Grundhoff et al., 1999) or the survival of motor neurons (SMN) protein (Barth et al., 2003; Voss et al., 2001). The binding of EBNA2 to a variety of other host proteins is reflected by its presence in high molecular weight complexes of different composition (Grässer et al., 1991; Tsui and Schubach, 1994; Wu et al., 2000). In mitotic cells, the transcriptional activity of EBNA2 is inhibited through phosphorylation at Serine 243 (Yue et al., 2005; Yue et al., 2006). Fig. 1 shows a schematic representation of EBNA2.

EBNA2 features an Arginine–Glycine (RG-) repeat element at positions 339–354 which contains symmetrically dimethylated

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**Fig. 1.** Schematic representation of the Epstein-Barr virus nuclear antigen 2 (EBNA2). EBNA2 of the standard B95.8 strain (accession number: AJ507799) of EBV consists of 487 amino acids (aa) present in A-type viruses. The N-terminal dimerisation domain ("Dim") is located next to a poly-proline stretch ("Pro"). The variable region ("variable") differs between the A-type viruses and B-type viruses. B-type viruses have a reduced *in vitro* transformation potential. The binding site for RBPJk ("RBPJk") is located around a Trp-Trp-Pro motif at aa 323–325. The adjacent Arginine–Glycine repeat ("ArgGly") between aa 339 and 354 confers binding to the survival of motor neurons (SMN) protein and represents the second nuclear localisation signal ("NLS") in addition to the canonical NLS found at the extreme C-terminus between aa 468 and 487. The C-terminal acidic transactivation domain ("TAD") between aa 424 and 468 interacts with various basal transcription factors.

Arginine (sDMA) residues that confer binding to the "Tudor" domain of the survival motor neuron protein (SMN) (Barth et al., 2003). EBNA2 might therefore represent the viral counterpart of the cellular Smd3 protein, which also associates with the Tudor domain of SMN via a symmetrically dimethylated RG repeat (Friesen and Dreyfuss, 2000). The deletion of the RG-repeat of EBNA2 results in a protein with a five-fold higher ability to stimulate expression of the viral oncogene LMP1 in reporter assays, but a recombinant virus featuring this deletion in EBNA2 has reduced transforming activity and needs an extended time span to induce transformed cell clones (Tong et al., 1994).

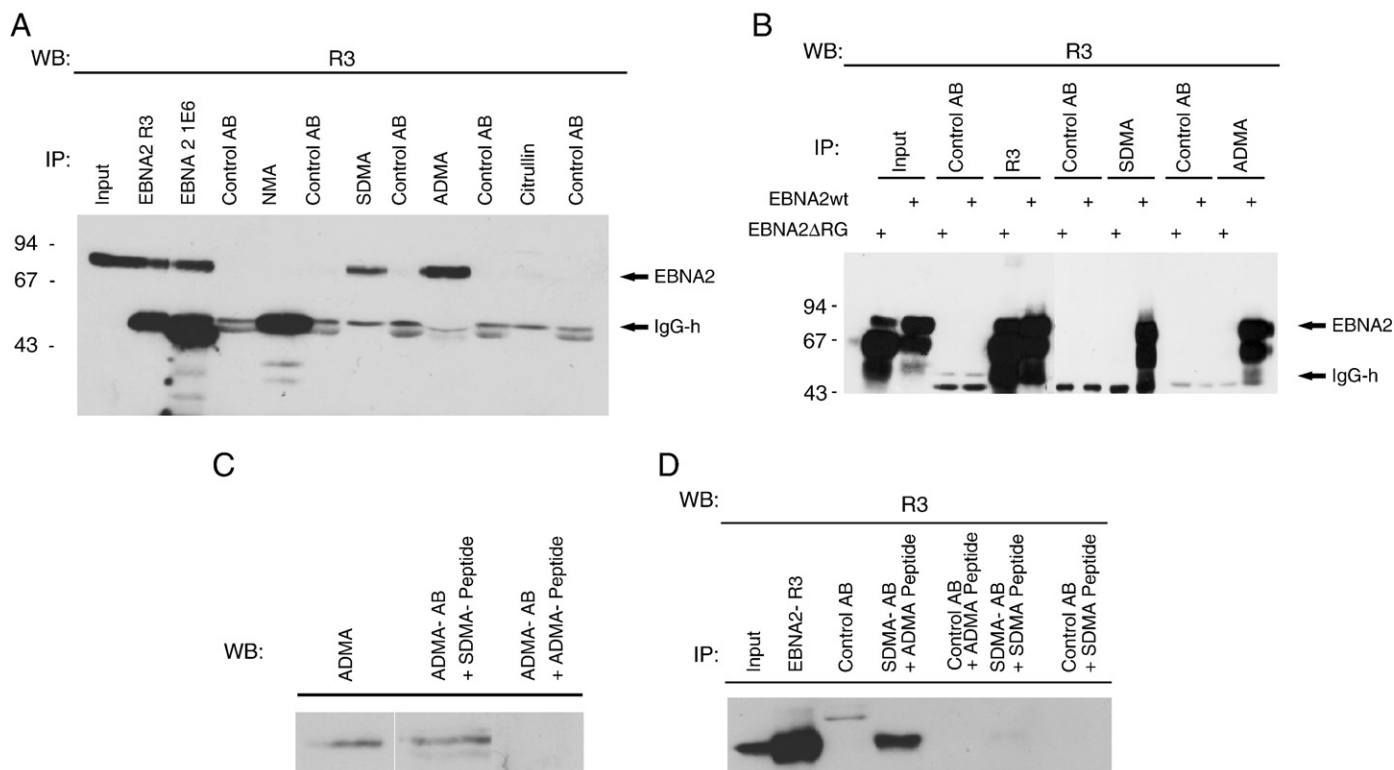
Methylation is a posttranslational modification that affects protein–protein interactions (Gary and Clarke, 1998) and plays a role in signal transduction, cellular proliferation, transcriptional processing and splicing of mRNA (Azzouz et al., 2005; Kim et al., 1997; Lee et al., 2005;

Stallcup et al., 2003). In addition to Lysine residues, methylation on proteins also takes place at Arginines (Paik and Kim, 1967) which leads to three known forms in higher eukaryotes:  $\omega$ -N<sup>G</sup>-MonoMethyl-Arginine (MMA),  $\omega$ -N<sup>G</sup>,N<sup>G</sup>-asymmetric DiMethyl-Arginine (aDMA) and  $\omega$ -N<sup>G</sup>,N<sup>G</sup>-symmetric DiMethyl-Arginine (sDMA); the methylation of the internal guanidino nitrogen atom to form  $\delta$ -N<sup>G</sup>-MonoMethylArginine has only been detected so far in yeast (for a recent review, see Bedford and Clarke, 2009). The methylation reactions are catalyzed by Protein-Arginine-Methyl-Transferases (PRMTs), which can be classified as type I enzymes (PRMT-1, -2, -3, -4, -6) which generate aDMA and type II enzymes (PRMT5, -7) which generate sDMA (for review, see Bedford and Richard, 2005). So far, JmjD6 is the only Arginine-demethylating enzyme with a demonstrated activity towards histone H3R2 and histone H4R3 (Chang et al., 2007). In addition, MMA- and aDMA-modified Arginines may be deiminated by the enzyme PAD14 to form citrulline residues (Cuthbert et al., 2004; Wang et al., 2004). Here we show using newly developed monoclonal antibodies (mAbs) that sDMA- and aDMA- but not MMA- nor citrulline-containing EBNA2 is present in EBV-infected cells. Most importantly, we demonstrate that asymmetrically Arginine dimethylated EBNA2 preferentially associates with DNA-bound RBPJk *in vitro* and with EBNA2-responsive promoters *in vivo*.

## Results

*EBNA2 contains either symmetrically or asymmetrically dimethylated residues within its Arginine–Glycine (RG-) repeat*

We had previously shown that the RG-repeat of EBNA2 contains sDMA-modified residues (Barth et al., 2003). To test whether EBNA2 also contains aDMA-modified, non-methylated Arginines or citrulline

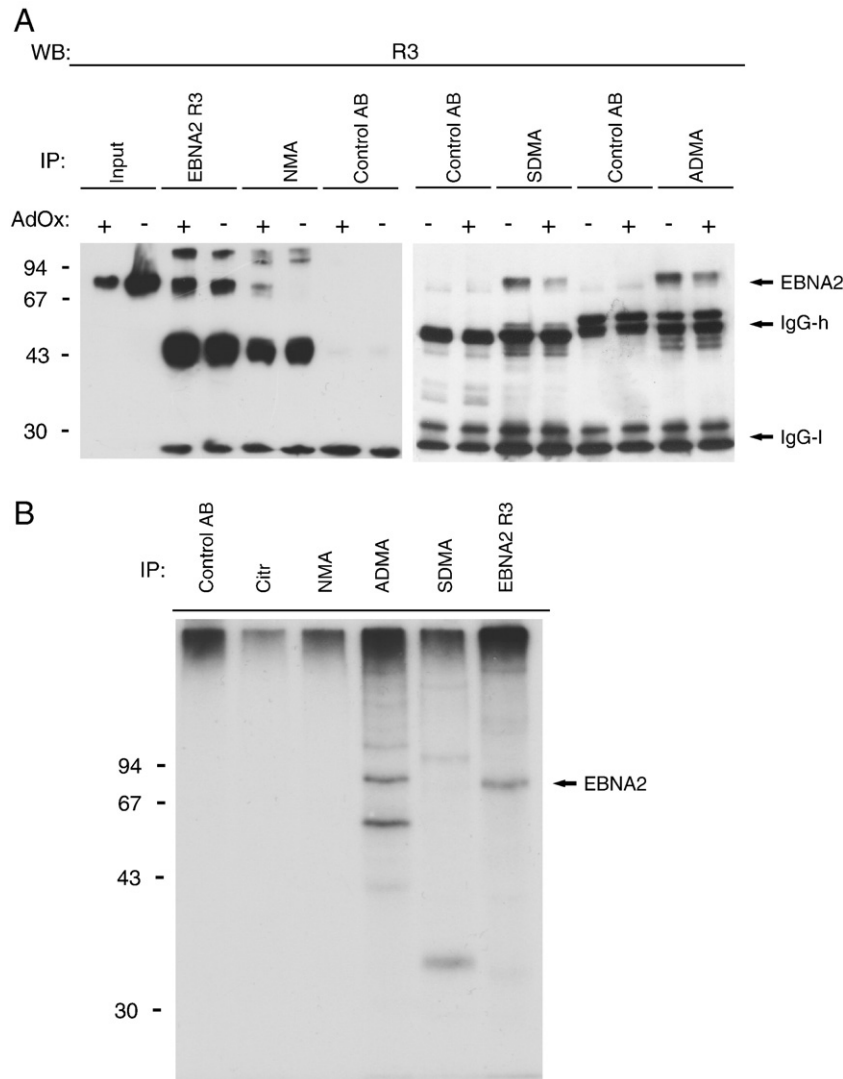


**Fig. 2.** Detection of methylated EBNA2 species. (A) Monoclonal antibodies (mAbs) directed against the non-methylated (NMA), sDMA-, aDMA-, or citrulline containing Arginine–Glycine (RG)-repeat of EBNA2 were tested by precipitation using extracts of EBV-positive B95.8 cells. For each antibody, an appropriate isotype control was tested in parallel to exclude unspecific binding to the protein G sepharose. Precipitated EBNA2 protein was visualised using the EBNA2-specific mAb R3 which binds outside the RG-domain of EBNA2. (B) Immunoprecipitation of EBNA2 from transiently transfected cells. HEK 293-T cells expressing either EBNA2-wt or the EBNA2-RG mutant were precipitated with R3 and the various methylation specific monoclonal antibodies as indicated using appropriate isotype control antibodies. The position of EBNA2 is indicated by an arrow. (C) Specific inhibition of aDMA-antibody by aDMA peptide. The aDMA-specific antibody 6F12 was used in a Western blot either untreated or pre-incubated with aDMA or sDMA-peptide as indicated. (D) Specific inhibition of sDMA-antibody by sDMA peptide. Extract of B95.8 cells was used for immunoprecipitation using either untreated or antibody 7D9 preincubated with aDMA or sDMA peptide. Irrelevant control antibody was used as an internal control. Precipitated EBNA2 was detected by Western blot using the R3 antibody.

residues instead of Arginines, we generated mouse or rat monoclonal antibodies (mAbs) against the various possible variations in its RG-repeat. KLH-coupled peptides corresponding to the non-methylated, symmetrically or asymmetrically dimethylated RG-repeat of EBNA2 or a peptide containing citrulline instead of Arginine residues were used for the immunisation, while the same peptides bound to OVA were then used in an ELISA screening assay (see Table S1, supplementary data). Only those antibodies that reacted exclusively with their cognate peptide were established as stable clones. The specificity of the antibodies was confirmed in a dot-blot assay (Figure S1, supplementary data). The antibodies were then tested by immunoprecipitation using extracts of EBV-positive B95.8 cells. The precipitated EBNA2 was detected using the previously described antibody R3 that binds to a C-terminal epitope outside the RG-repeat (Kremmer et al., 1995). As shown in Fig. 2A, only the sDMA and aDMA-specific but not the NMA- or citrulline-specific antibodies yielded a signal for EBNA2 indicating that both methylated forms are present in EBV-infected cells. EBNA2 was then immunoprecipitated from extracts of 293-T cells transiently expressing either EBNA2-wt or the mutant EBNA2- $\Delta$ RG with a deletion of the RG repeat (Tong et al.,

1994). As shown in Fig. 2B, the sDMA and aDMA-specific antibodies reacted with EBNA2-wt but not the deletion mutant demonstrating that the correct epitope on EBNA2 was recognised. Moreover, the signal for EBNA2 could be inhibited by preincubation of the 6F12 antibody with the aDMA-containing peptide but not the sDMA-peptide demonstrating again that the antibody is specific for aDMA-EBNA2 (Fig. 2C). Vice versa, we were able to inhibit the immunoprecipitation of EBNA2 by the sDMA-specific antibody 13B10 using the sDMA peptide but not the aDMA-peptide (Fig. 2D).

EBV-positive B95.8 cells were then treated with the methylation inhibitor AdOx prior to immunoprecipitation of EBNA2. As can be seen in Fig. 3A, the overall amount of EBNA2 remained essentially unchanged while the signal obtained with the sDMA- or aDMA-specific antibodies decreased. In addition, we now detect a signal for EBNA2 with the NMA-specific antibody. We observed additional immunoreactive bands above the EBNA2 signal which might be derived from IgG molecules that contained incompletely reduced disulfide bonds. The appearance of unmethylated EBNA2 in the AdOx-treated cell extract confirms the specificity of the NMA-antibody. Our data demonstrate that EBNA2 does not exist in a non-



**Fig. 3.** (A) Inhibition of methylation by AdOx treatment. EBNA2 was precipitated with the indicated antibodies from B95.8 cell extract treated with the methylation inhibitor AdOx. Precipitated EBNA2 was visualised using the R3 antibody. The IgG heavy ("IgG-h") and light ("IgG-l") chains of the antibodies released from the beads were also detected by the secondary peroxidase coupled anti-rat antibody. Co-electrophoresed molecular mass marker proteins ( $\times 10^{-3}$  kDa) were, in descending order: phosphorylase B, bovine serum albumin (BSA), ovalbumin (OVA), and carboanhydrase. (B) Only aDMA-EBNA2 is phosphorylated. The different antibodies as indicated were used to precipitate EBNA2 from  $^{32}$ P-labelled B95.8 cell extracts. The bound EBNA2 was analysed by SDS-PAGE and autoradiography. The position of EBNA2 is indicated. Co-electrophoresed  $^{14}$ C-labelled molecular mass marker proteins ( $\times 10^{-3}$  Da) were, in descending order: phosphorylase B, bovine serum albumin (BSA), ovalbumin (OVA), and carboanhydrase.

methyated state or in a form that has the Arginines in the RG-repeat converted to citrullines. Most importantly, the antibody 6F12 (see below) developed against a peptide containing aDMA reacted with EBNA2 indicating that a substantial fraction contains aDMA in addition to the sDMA-modified EBNA2 establishing aDMA as a novel modification of EBNA2. We obtained comparable signals for sDMA- vs. aDMA-containing EBNA2 but since the antibodies used may display different affinities for their antigens, this does not provide absolute quantification of the relative abundance of the two methylated forms.

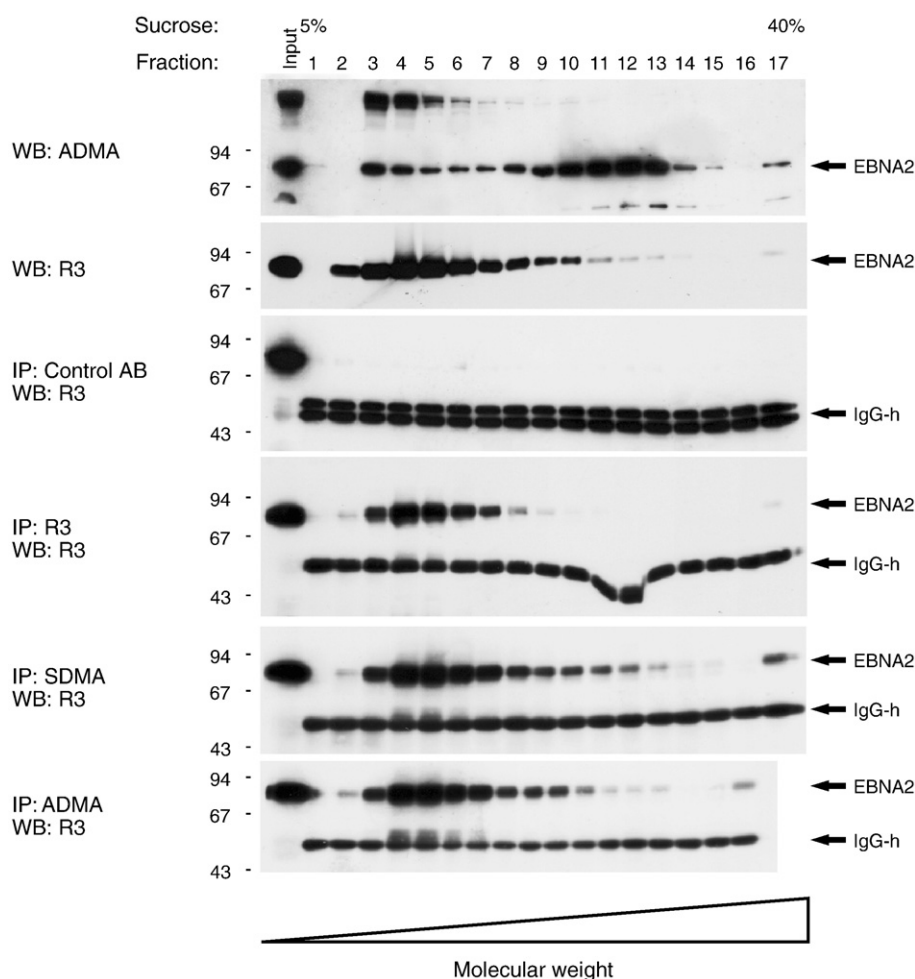
#### Only ADMA-modified EBNA2 is phosphorylated

EBNA2 is a phosphoprotein (Grässer et al., 1992; Petti et al., 1990). Hyperphosphorylation was shown to inhibit transcriptional activation of EBNA2 during mitosis (Yue et al., 2004). To test whether the aDMA- or sDMA-modified EBNA2 are phosphorylated, EBV-positive B95.8 B-cells were metabolically labelled with  $H_3^{32}PO_4$  and the cell extract was immunoprecipitated with the different antibodies and subsequently analysed by SDS-PAGE and autoradiography (Grässer et al., 1991). As can be seen in Fig. 3B, the precipitation with the aDMA-specific antibody as well as the R3-antibody but not the sDMA-, NMA- or citrulline-specific antibodies yielded signals for EBNA2. This data indicates that only the aDMA-modified EBNA2 is detectably phosphorylated. We noticed additional bands co-precipitated by the aDMA- and sDMA-specific antibodies. As EBNA2 forms high-molecular

weight complexes, these phosphoproteins might represent factors associated with either aDMA or sDMA-modified EBNA2. The bands that were precipitated with the aDMA-specific antibody migrated to different positions in the gel than those precipitated by the sDMA-specific antibody indicating that the sDMA and aDMA forms of EBNA2 associate with different cellular proteins. We also tested whether the induction of lytic EBV-replication by TPA influences the methylation status of EBNA2 as it was shown that phosphorylation of Serine 243 by the viral kinase BGLF4 was induced during lytic cycle replication (Yue et al., 2005). The induction of the lytic cycle was confirmed using recently developed antibodies against the viral DNA polymerase (Barth et al., 2008) or against the BZLF1 protein (Young et al., 1991). We found no change in the methylation of the protein upon lytic cycle induction by TPA-treatment (see supplemental Figure S2A), while the AdOx-treatment reduced the methylation of EBNA2 but did not induce the lytic cycle (see supplemental Figure S2B).

#### aDMA and sDMA-containing EBNA2 is detectable in high-molecular weight complexes

EBNA2 forms high molecular weight complexes consistent with its association with multiple proteins (Grässer et al., 1991; Wu et al., 1996). Nuclear extracts were separated by 5–40% sucrose gradient centrifugation (Grässer et al., 1991) and tested in a Western blot using the antibody R3 or the aDMA-specific clone 6F12. As shown in Fig. 4,



**Fig. 4.** Methylated EBNA2 is present in high molecular weight complexes. Native cell extracts were subjected to 5–40% sucrose gradient centrifugation. The fractions obtained were either concentrated by ethanol precipitation and then analysed by Western blot analysis with the indicated antibodies (panels labelled “WB”) or subjected to precipitation followed by Western blot analysis (panel labelled “IP/WB”) with the indicated antibodies. The GST-specific mAb 6G9 (Kremmer, unpublished) was used as a control for the precipitation. The bands at approx. 50 kDa represent the heavy chains of the antibodies used for precipitation that are detected by the secondary peroxidase-coupled anti-rat antibody. Molecular mass marker proteins were described in Fig. 2.

the majority of EBNA2 detectable by R3 migrated in fractions 3–6 representing dimeric EBNA2 in complexes of about 300–400 kDa. In contrast, the aDMA-specific clone 6F12 yielded the strongest signal in fractions 10–13 corresponding to a molecular mass of about 500–700 kDa and a distinct signal in the bottom fraction 17 corresponding to a very high molecular mass. Extreme care was taken to avoid resuspending particulate material that might have sedimented at the bottom of the centrifuge tubes. Tsui and Schubach (1994) have described an EBNA2 species of 3–4 MDa detectable in gel filtration experiments which is consistent with the EBNA2 species present in fraction 17. Using 6F12 (aDMA antibody), we also observed an immunoreactive band of approx. 200 kDa in fractions 3–6 that might represent dimeric EBNA2 as the samples were not boiled prior to the gel electrophoresis. Because the sDMA-specific antibodies did not work in Western blot analysis, we subjected the fractions of the gradients to an immunoprecipitation employing EBNA2-R3, the sDMA- or aDMA-specific antibodies and an irrelevant control antibody. The precipitated EBNA2 was visualised using R3. As can be seen in Fig. 4, the control antibody gave no signal, while the precipitation with R3 and the other antibodies yielded signals with a peak in fractions 3–6. R3 and the aDMA- and sDMA-specific mAbs also precipitated higher molecular weight species (fraction 17). The major part of aDMA-containing EBNA2 sediments in the fractions 10–13 corresponding to the higher molecular weight complexes while the precipitation mainly yielded a signal in the fractions 3–6 for the lower molecular mass complexes. This result indicates that the former complexes contain proteins bound to the modified RG-repeat which render this epitope inaccessible to the antibody by immunoprecipitation. Because we clearly detect some EBNA2 precipitated from fraction 17 presenting very high molecular weight complexes, we assume that sDMA-containing EBNA2 is also present in the fractions 10–13 but cannot be precipitated because of RG-bound proteins.

#### *Only aDMA-containing EBNA2 is detectable in DNA-binding complexes in vitro*

We next tested for the presence of methylated EBNA2 in DNA-associated complexes. For this purpose, we analysed the ability of the methylation-specific antibodies to induce a supershift in an electrophoretic mobility shift assay (EMSA). We had originally shown that EBNA2 is tethered via RBPJ $\kappa$  to its cognate promoter sequences (Zimber Strobl et al., 1993). It is important to point out that although considerable evidence, including data from chromatin immunoprecipitation experiments (Bark-Jones, Webb and West, 2006), supports the fact that EBNA2 stably associates with DNA via RBPJ $\kappa$ , we and others have found that EBNA2 can destabilise the single RBPJ $\kappa$ -DNA complex detected in gel retardation assays carried out using C promoter probes containing one RBPJ $\kappa$  binding site (Waltzer et al., 1994, 1996 and our unpublished data). It is therefore important to ensure that the *in vitro* assays used accurately reflect the behaviour of RBPJ $\kappa$  and EBNA2 *in vivo*. Using an LMP 2A/TP1 promoter probe containing two RBPJ $\kappa$  sites allows the detection of the RBPJ $\kappa$  and/or EBNA2-containing complexes: complex I represents one molecule of RBPJ $\kappa$  bound to DNA, complex III corresponds to two molecules of RBPJ $\kappa$ , and complex IV contains two molecules of RBPJ $\kappa$  and one molecule of EBNA2, while cell proteins non-specifically bound to the promoter are present in complex II. Complex IV can be “supershifted” by R3 (Meitinger et al., 1994). Conversely, the interaction of EBNA2 with the DNA-bound RBPJ $\kappa$  can be destroyed by the antibody 6C8 which recognises the “WWP”-motif of EBNA2 necessary for binding to RBPJ $\kappa$  (Sauder et al., 1996). Prior experiments had shown that EBNA2 generated in a reticulocyte-based system was able to induce an EBNA2-specific shift that could be supershifted with R3 antibody and was also destroyed by the 6C8 antibody as was observed for EBNA2 from native cell extracts (Meitinger et al., 1994; Sauder et al., 1994; Zimber Strobl et al., 1993). In addition, cell extract as a source for

RBPJ $\kappa$  can also be substituted by IVT-RBPJ $\kappa$  from the reticulocyte system (Maier et al., 2005). The *in vitro* generated EBNA2 reacted with the antibody 6F12 (aDMA) in a Western blot and was also precipitated using the sDMA-specific antibody 7D9 indicating that both sDMA and aDMA modified EBNA2 were generated (Figure S3, supplementary data) in line with our previous report that the reticulocyte-based EBNA2 contains sDMA residues (Barth et al., 2003). As can be seen in Fig. 5A, EBNA2 alone did not bind to the probe (lane 2) while the IVT-RBPJ $\kappa$  yielded a band (lane 3) that could be shifted with IVT-EBNA2 (lane 4) and could furthermore be supershifted by R3 (lane 5). The WWP mAb 6C8 destroyed this complex (lane 6). Significantly, only the aDMA-specific clone generated a supershift (lane 11), while neither the NMA-, the sDMA- nor the control antibodies bound to the RBPJ $\kappa$ -EBNA2 DNA bound complex (lanes 7–10). The same result was also obtained when gel shift experiments were carried out using extracts from EBV-infected cells (Fig. 5B). Here, the R3 antibody supershifted complex IV (Fig. 5B, lane 4) which was destroyed by 6C8 (lane 5), while only the aDMA-specific antibody induced a supershift (lane 10). Moreover, when reticulocyte-derived EBNA2 was used in conjunction with cell extract derived from the EBNA2-deficient, EBV-positive P3HR1 cell line, we again obtained an EBNA2-specific band that was only supershifted by R3 and the aDMA-specific clone 6F12, but not by the sDMA- or NMA-specific antibodies (data not shown). These data therefore support the notion that the aDMA-modification of EBNA2 is necessary to promote the association of EBNA2 with DNA-bound RBPJ $\kappa$ . To exclude the possibility that the gel shift buffer conditions prevented binding of the sDMA-specific antibody to its epitope, immunoprecipitations were carried out using the same nuclear extracts and conditions. We could clearly precipitate EBNA2 with the sDMA-specific antibody (supplementary Figure S4A). Furthermore, we found that the antibodies still bound to their OVA-conjugated peptides in a dot-blot assay when the blot strips were incubated with up to 2 M NaCl excluding grossly different binding affinities as an explanation for the observed results (data not shown).

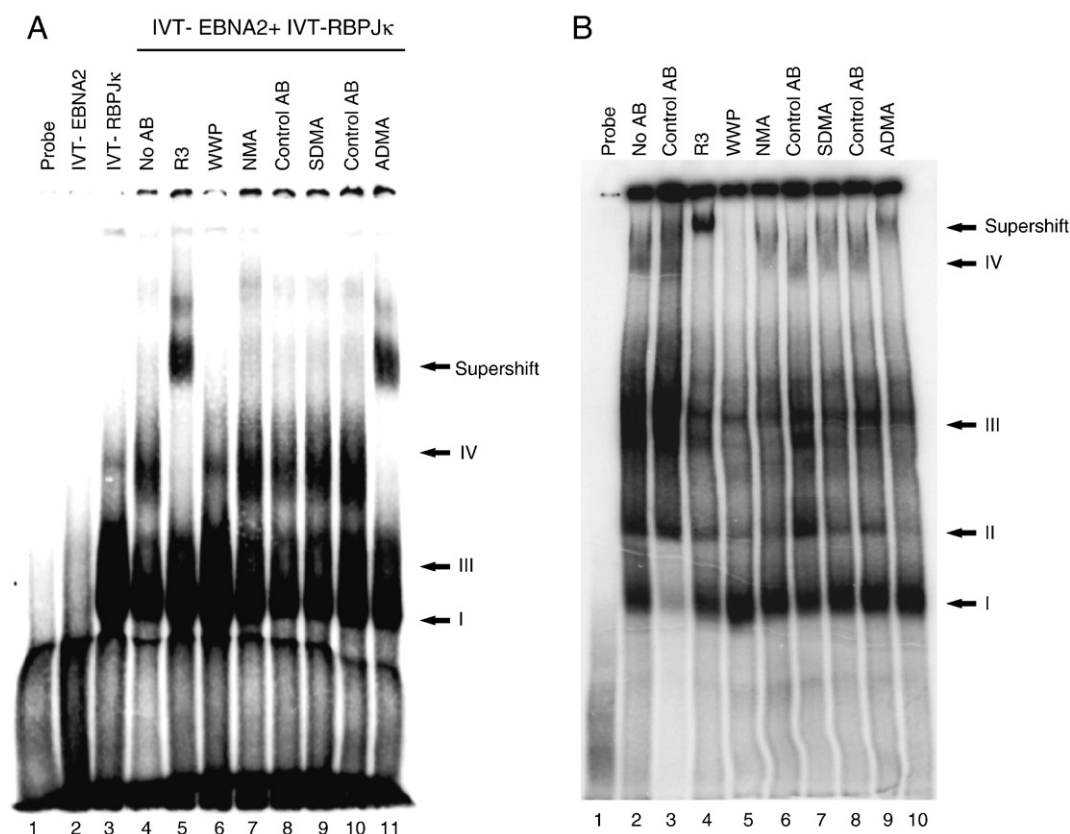
#### *Methylation is a prerequisite for interaction of EBNA2 with DNA-bound RBPJ $\kappa$*

Next, we analysed nuclear extracts of cells treated or untreated with the methylation inhibitor AdOx in a gel shift assay. We observed a decrease in methylation of EBNA2 in these extracts while the level of the protein was unaffected (see above). As can be seen in Fig. 6A, the EBNA2-derived shift observed with untreated cell extract was stronger than the one obtained with the AdOx-treated extract demonstrating again that methylation of EBNA2 influences its interaction with DNA-bound RBPJ $\kappa$ . The determination of the signal strength showed a decrease in signal intensity of 30–67% for the AdOx-treated samples (compare, i.e., lanes 2 and 3). Again, we only observed a supershift with R3 (lanes 6 and 7) and the aDMA-specific antibody (lanes 18 and 19). Here, untreated extract showed a supershift with the aDMA antibody (lane 18) while essentially no supershift with this antibody was detectable with the AdOx-treated sample (lane 19). For better clarity, the upper part of the gel depicted in Fig. 6A as well as a quantification of the signal from the supershift of the treated and the corresponding untreated samples is shown in Fig. 6B.

#### *Methylation converts non-binding EBNA2 into a DNA-associated form*

We then assayed the DNA-binding properties of non-methylated EBNA2 produced in an *E. coli*-based transcription-translation system (“*E. coli* E2”). By precipitation with the NMA-specific antibody we could show that the *E. coli*-based EBNA2 was indeed not methylated and initial experiments indicated that the *E. coli*-EBNA2 did not bind to DNA (data not shown). To determine whether methylation of EBNA2 by a methyl transferase activity in the reticulocyte system converted EBNA2 into the RBPJ $\kappa$ -associated, DNA-binding form, we





**Fig. 5.** aDMA-modified EBNA2 is present in DNA-binding complexes. (A) EBNA2 and RBPJk synthesised in a coupled *in vitro* transcription–translation system derived from rabbit reticulocytes (Promega) were used in a gel shift (EMSA) (Meitinger et al., 1994) after preincubation of the proteins with the indicated antibodies. The complexes III and IV which are formed by RBPJk and RBPJk plus EBNA2, respectively, as well as complex IV supershifted with mAb R3 (lane 4) or aDMA-specific mAb 6F12 (lane 11) are indicated. (B) EBNA2-containing Raji cell extract was incubated with the indicated antibodies and then assayed in a gel shift assay. R3 recognises EBNA2 regardless of its methylation status and induces a “supershift” indicated by the upper arrow (Zimber Strobl et al., 1993), the mAb 6C8 directed against the “WWP”-repeat of EBNA2 destroys the EBNA2/RBPJk-complex IV (Sauder et al., 1994). Antibodies used are indicated above each lane. Control antibodies corresponded to the respective IgG-subtype of each antibody. To efficiently separate the high molecular weight complexes, the electrophoresis was carried out for an extended time. Therefore, uncomplexed  $^{32}$ P-labelled probe ran out of the gel. The position of the RBPJk-containing complexes I–IV as described in the text are indicated; the arrow points at the EBNA2-containing complex IV that is supershifted by R3 and aDMA-6F12 but destroyed by WWP-6C8.

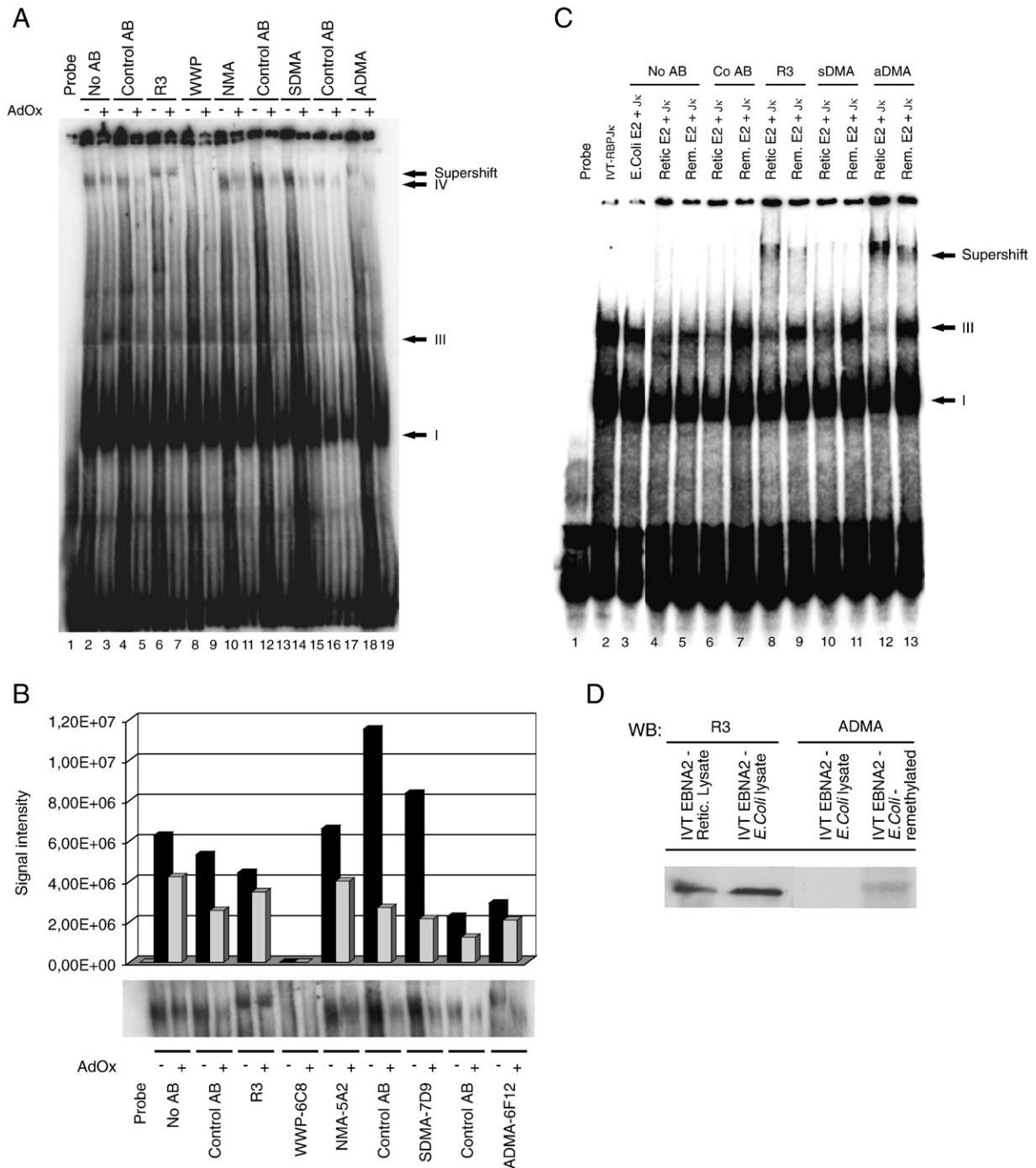
incubated the *E. coli*-EBNA2 with the un-programmed reticulocyte-based transcription–translation extract supplemented with the methyl donor SAM and carried out the EMSA using this remethylated-EBNA2 (“Rem. E2”) in conjunction with IVT RBPJk. Gel shift assays were carried out with the same amount of *E. coli*-EBNA2 as the reticulocyte-EBNA2 (“Retic E2”) and IVT-RBPJk. The *E. coli*-EBNA2, the remethylated-EBNA2 or the reticulocyte-EBNA2 did not produce a supershift alone (Fig. 6C, lanes 3, 4 and 5, respectively). The remethylated and the reticulocyte EBNA2 were then either assayed with control-antibody (lanes 6 and 7, respectively), R3 (lanes 8 and 9), the sDMA-specific antibody (lanes 10 and 11) or aDMA-specific antibody (lanes 12 and 13). As can be seen, only R3 and the aDMA- but not the sDMA-antibody induced a supershift. The NMA-specific antibody also did not generate a supershift (Figure S5, supplementary information). Western blot analysis (Fig. 6D) confirmed that the C-terminal antibody R3 recognised EBNA2 from both the reticulocyte- and the *E. coli*-based IVT system while the aDMA-specific antibody 6F12 only reacted with the remethylated-EBNA2 but not the *E. coli*-EBNA2 showing that aDMA-EBNA2 can be generated *in vitro*.

To determine whether demethylation of EBNA2 *in vivo* results in a reduction in EBNA2-activated transcription of its target promoters, we investigated the expression of the EBNA2-responsive LMP1 gene. EBV-infected B95.8 monkey cells or the human IB4 lymphoblastoid cell line immortalised with B95.8 virus were treated with AdOx. Although the overall amount of EBNA2 was only reduced by 10–20%, we observed a 70% reduction in the amount of aDMA-EBNA2 detected by Western blot and a corresponding decrease in the expression of

LMP1 by 40–50% while the  $\beta$ -actin loading control was unaffected (Fig. 7). Comparable results were obtained using Raji cells (data not shown). These results support the notion that methylation is a prerequisite for binding of EBNA2 to DNA.

#### aDMA modified EBNA2 preferentially binds to promoters *in vivo*

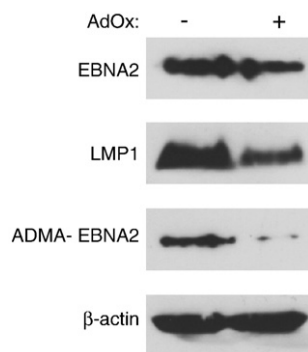
To investigate the effect of EBNA2 methylation on the binding of EBNA2 to promoters *in vivo*, we carried out chromatin immunoprecipitation experiments using a matched pair of EBNA2 negative and positive Burkitt's lymphoma cell-lines. The group I Burkitt's lymphoma (BL) cell-line Mutu I was derived from a tumour biopsy and maintains the characteristic restricted form of latency (latency I) found in BL, associated with the expression of only EBNA 1. The Mutu III cell-line was derived from Mutu I cells that had drifted in culture to express the full panel of EBV latent genes including EBNA2 (latency III). These cell-lines therefore allow an examination of the transcriptional effects of EBNA2 in the same cell background. The presence or absence of EBNA2 in the two cell lines was confirmed by Western blot analysis (supplemental Figure S6 and data not shown). Primers were designed to amplify three EBNA2 target EBV promoters; the C promoter (Cp), the LMP1 promoter (LMP1p) and the LMP2A promoters (LMP2Ap) (Figs. 8A–C, respectively). Using the R3 antibody, we observed the expected profile of EBNA2 binding to Cp, peaking in the region encompassing the RBPJk site (–368 to –374, Waltzer et al., 1994) detected by the –430 to –355 amplicon (–430) (Fig. 8D). These results were in line with our previous observations using the anti-



**Fig. 6.** Methylation of EBNA2 is a prerequisite for DNA-binding. (A) Extracts of Raji cells either treated (lanes designated “+”) or untreated (lanes designated “–”) with the methylation inhibitor AdOx were used in the gel shift experiments. The antibodies used are given above each lane. The upper arrow indicates the supershifted EBNA2-containing complex IV. (B) The figure shows the quantification upper section of the supershift shown in Fig. 5A together with the quantification of the supershifted EBNA2-containing complex obtained using the PhosphorImager® (GE Healthcare, Freiburg, Germany). (C) Remethylation generates DNA-binding EBNA2. The DNA probe (lane 1) was either incubated with *in vitro* generated RBPJk alone (“IVT RBPJk”, lane 2), with IVT-RBPJk plus unmethylated *E. coli*-EBNA2 alone (“*E. coli* E2 + Jk”, lane 3) or remethylated *E. coli*-EBNA2 (“Rem. E2”) together with *in vitro* synthesised RBPJk (lane 4). The indicated combinations of *E. coli*-EBNA2, remethylated EBNA2 or Reticulocyte-based EBNA2 (“Retic E2”) were tested with IVT-RBPJk and control-antibody (“Co AB”, lanes 6 and 7), EBNA2-specific R3 antibody (“R3”, lanes 8 and 9), sDMA-specific antibody (“sDMA”, lanes 10 and 11) or the aDMA-specific antibody (“aDMA”, lanes 12 and 13). The shifted and supershifted EBNA2-containing complexes are indicated. (D) aDMA modification of EBNA2 by *in vitro* methylation. EBNA2 was synthesised *in vitro* in the *E. coli*-based RTS 500 system (Roche). This unmethylated *E. coli*-EBNA2 was then pre-incubated with unprogrammed reticulocyte lysate. The left lane contains reticulocyte-derived EBNA2 (“IVT-EBNA2 Retic. Lysate”) and the adjacent lane features *E. coli*-synthesised EBNA2 (“IVT-EBNA2 *E. coli* lysate”) as detected by the EBNA2-specific R3 antibody. The next lane contains unmethylated *E. coli*-EBNA2 (“IVT-EBNA2 *E. coli* lysate”), and the rightmost lane contains *E. coli*-EBNA2 remethylated with the reticulocyte lysate (“IVT-EBNA2 *E. coli*-remethylated”). In these two lanes, EBNA2 was stained using the aDMA-specific antibody 6F12.

EBNA2 PE2 mouse mAb (Bark-Jones, Webb and West, 2006). The aDMA specific antibody detected high levels of EBNA2 binding consistent with the presence of aDMA-EBNA2 in promoter-bound complexes (Fig. 8E). In contrast, the sDMA specific antibody detected

significantly less binding of sDMA-modified EBNA2 to the promoter. Similar results demonstrating comparatively less association of sDMA-modified EBNA2 with promoter sequences were obtained using LMP1 and LMP2A promoter primers, with the peak of EBNA2



**Fig. 7.** Inhibition of EBNA2 methylation reduces LMP1 expression. B95.8 cells were untreated (“–”) or treated (“+”) with the methylation inhibitor AdOx and analysed by Western blot for the expression of EBNA2, LMP1, aDMA-EBNA2. Levels of EBNA2 (“EBNA2”, top panel), LMP1, aDMA-modified EBNA2 and  $\beta$ -actin as loading control were determined using the antibodies R3, S12, 6F12 and AC15 (Sigma), respectively.

binding detected by the primer sets encompassing one or more of the RBPJ $\kappa$  sites (Figs. 8F–I). Control experiments confirmed that both aDMA and sDMA-specific antibodies were able to precipitate equivalent amounts of EBNA2 protein from cross-linked chromatin under ChIP conditions (supplemental Figure S4B), indicating that the reduced signal obtained in the real-time PCR analysis reflects a decreased association of sDMA-EBNA2 with promoter DNA. These data therefore indicate that aDMA-modified EBNA2 is likely to represent the predominant form of EBNA2 found at target promoters *in vivo*.

## Discussion

Here, we provide the first demonstration of aDMA-methylation as a novel modification of EBNA2. In addition, essentially all EBNA2 molecules in the cell are converted into methylated forms and citrulline-containing EBNA2 was not detectable. Our data show that methylation influences the interaction of EBNA2 with RBPJ $\kappa$  and implicate methylation in the regulation of the activity of this oncoprotein. It has been demonstrated that histones undergo various secondary modifications including Arginine methylation (Jenuwein and Allis, 2001). In addition to methylation at Lysine residue 7, histone H3 may be methylated at Arginine 8 by either PRMT5 or PRMT1. The latter modification was shown to activate transcription, while methylation by PRMT5 inactivated transcription (Pal et al., 2004 and references therein). We assume that PRMT1 generates aDMA-modified histone H3, while PRMT5 produces sDMA-histone H3. No clear picture has emerged for methylation of transcription factors. For example, aDMA-STAT1 generated by PRMT1 (Mowen et al., 2001) as well as sDMA-IL-2 generated by PRMT5 (Richard, Morel, and Cleroux, 2005) were both activating. We have not formally shown that EBNA2 is methylated by PRMT1 but it is likely that either PRMT1 or another type I methyltransferase is responsible for the generation of aDMA-containing EBNA2. In our assays, we find predominantly aDMA-modified EBNA2 at promoters *in vivo*, indicating that type I methyltransferases produce a positive effect on transcription in this context.

The data obtained from our ChIP analyses indicated that less sDMA-modified EBNA2 associated with three EBV target promoters *in vivo*, compared to the signals obtained using the R3 or aDMA-specific antibodies. These data indicate that the aDMA-modified form of EBNA2 preferentially associates with promoters *in vivo*. Nonetheless, these ChIP experiments do detect some sDMA association with promoters in contrast with the results of the gel shift experiments where the sDMA-specific antibody was not able to supershift an EBNA2-RBPJ $\kappa$  DNA complex at all. It is possible that the sDMA antibody epitope is occluded in the EBNA2-RBPJ $\kappa$ -DNA complex formed *in vitro*, possibly by other

factors present in the complex. The association of EBNA2 and/or RBPJ $\kappa$  with the additional components of the transcriptional machinery present at promoters *in vivo* may result in the release of these factors or induce a conformational change in the complex that re-exposes the epitope. It is also possible that aDMA-EBNA2 binds to RBPJ $\kappa$  directly while sDMA-EBNA2 associates with other factors present at the promoters *in vivo*.

We have demonstrated previously that EBNA2 targets the survival of motor neurons (SMN) protein via its RG-repeat modified by the type II PRMT5 (Barth et al., 2003) and that co-expression of SMN activates transcription by EBNA2 (Voss et al., 2001). However, a ChIP analysis using antibodies against SMN did not yield a signal from the LMP1 promoter although SMN was clearly precipitated (data not shown). It is still unclear why the deletion of the RG-repeat reduces transformation by EBNA2 while the expression of the viral oncogene LMP1 is strongly up-regulated. One possibility is that the deletion of the RG-repeat without insertion of a spacer to replace the deleted amino acids results in an altered EBNA2 protein that is able to interact with RBPJ $\kappa$  while the presence of the non-methylated RG-repeat inhibits association with DNA-bound RBPJ $\kappa$ . This data is in line with our finding that inhibition of EBNA2 methylation results in a decrease of LMP1 protein synthesis.

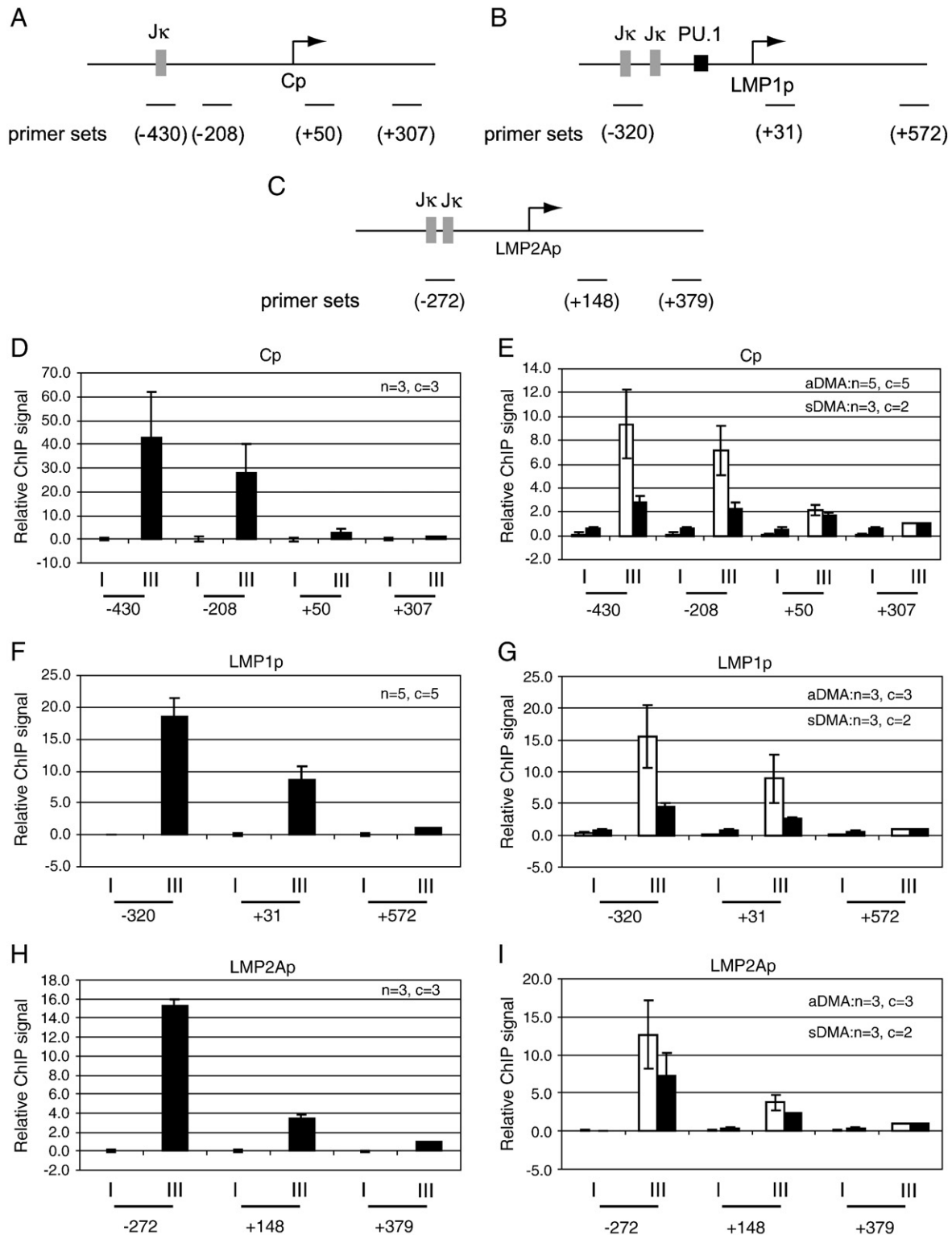
Interestingly, although AdOx treatment reduced the level of methylated EBNA2, it did not completely ablate it. It is unclear whether EBNA2 undergoes demethylation (deimination) within its RG-repeat as we saw no citrulline-containing EBNA2 with our antibodies. Because the half-life of EBNA2 is very long (Grässer et al., 1991), it is unlikely that proteolytic degradation of EBNA2 removes “active” EBNA2 from the cell. However, DNA-bound aDMA-EBNA2 was phosphorylated, while the sDMA-EBNA2 was not. It is possible that during mitosis, as shown by others (Yue et al., 2004), the DNA-bound aDMA-EBNA2 is reversibly inactivated through phosphorylation at Ser243.

In summary, we show that EBNA2 contains both sDMA and aDMA-residues and that methylation is required for DNA-binding by EBNA2, with aDMA-modified EBNA2 preferentially associated with promoters *in vitro* and *in vivo*. In addition, only aDMA-containing EBNA2 is phosphorylated pointing at further functional differences between these subspecies of EBNA2. Further studies will be necessary to determine how differential methylation of EBNA2 regulates its association with the components of the transcriptional machinery and affects its biological activities.

## Materials and methods

### Generation of monoclonal antibodies (mAbs)

Lou/C rats or BALB/c mice were immunised with KLH-coupled peptides corresponding to the Arginine–Glycine repeat of EBNA2 that contained either non-methylated Arginines (NMA), symmetrically (sDMA) or asymmetrically (aDMA) dimethylated Arginine residues or a peptide containing citrulline instead of Arginine residues. The peptides were based on the sequence NH<sub>2</sub>-C-GQSRGRGRGRGRGK GKSRDK; the amino terminal, non-EBNA2-derived Cysteine residue was added for covalent coupling to KLH or OVA. The screening was carried out by ELISA with the peptides coupled to OVA using an irrelevant peptide as a control (Barth et al., 2008). Positive clones were then tested against the other peptides and only those that reacted exclusively with their cognate peptide were established. KLH- and OVA-coupled peptides were purchased from PSL, Heidelberg, Germany. The rat mAb 8C12 (IgG2a) reacts with NMA-EBNA2, the mouse mAb 13B10 (IgG2a) recognises sDMA-EBNA2, the mouse mAb 6F12 (IgG2b) binds to aDMA-EBNA2 and the rat antibody 4A6 (IgG1) reacts with citrulline-containing EBNA2-derived peptide. The antibodies R3, 1E6 and 6C8 were described previously (Kremmer et al., 1995). R3 and 1E6 bind to a C-terminal epitope, 6C8 recognises the WWP-repeat at aa 320 and destroys the EBNA2-RBPJ $\kappa$ -interaction



**Fig. 8.** Chromatin immunoprecipitation analysis. Location of the amplicons generated by the C promoter (A) or LMP1 promoter (B) or LMP2A promoter (C) real-time PCR primer sets. Numbers refer to the start of the amplicons generated by the indicated primer sets relative to the transcription start sites. The RBPJκ and PU.1 binding sites are indicated by grey and black boxes, respectively. The LMP1 promoter sequence has been inverted for simplicity and lies in the reverse orientation in the EBV genome. (D) Chromatin was immunoprecipitated from Mutu I (I) or Mutu III cells (III) using the R3 rat mAb and analysed using Cp-specific primers. To allow comparison between antibodies and experiments relative ChIP signals were calculated by expressing the percentage input signal relative to the Mutu III signal obtained with the furthest downstream primer set. Results show the mean  $\pm$  standard deviation for at least three independent experiments (*n*) carried out on at least two different batches of chromatin (*c*). (E) Chromatin immunoprecipitations carried out using the aDMA EBNA2 specific (6F12, open bars) and sDMA EBNA2-specific (7D9, black bars) mouse mAbs, analysed using Cp specific primers. (F) Chromatin immunoprecipitations carried out using the R3 rat mAb analysed using LMP1p-specific primers. (G) Chromatin immunoprecipitations carried out using the aDMA-EBNA2 and sDMA EBNA2-specific mouse mAbs analysed using LMP1p specific primers. (H) Chromatin immunoprecipitations carried out using the R3 rat mAb analysed using LMP2Ap-specific primers. (I) Chromatin immunoprecipitations carried out using the aDMA-EBNA2 and sDMA EBNA2-specific mouse mAbs analysed using LMP2Ap specific primers.



(Sauder et al., 1994). S12 binds to LMP1 (Mann et al., 1985), the monoclonal antibody 3F10 (Roche, Penzberg, Germany) binds to the HA-tag. Mouse AC-15 anti  $\beta$ -actin was from Sigma (München, Germany).

#### Dot blot assay

Approx. 0.1  $\mu$ g each of the OVA-coupled peptides (see above) dissolved in PBS was spotted onto a nitrocellulose membrane (Whatman Protran®, Dassel, Germany). OVA with HA-peptide reactive with the HA-specific antibody 3F10 served as an internal control. The membrane strips containing the different peptide conjugates were blocked for 30 min at 25 °C with 1% non-fat dried milk dissolved in PBS, and then incubated with the different antibodies diluted 1:10 in the milk/PBS solution overnight. The bound antibodies were visualised by the ECL®-method (GE Healthcare, München, Germany) as described (Barth et al., 2003) using goat-anti-rat or -anti-mouse-antibody coupled to horseradish peroxidase as secondary antibody.

#### Cell lines and tissue culture

HEK 293-T cells were cultured in DMEM medium (GIBCO), supplemented with 10% FCS and antibiotics, non-adherent cell lines were grown in RPMI 1640 medium (GIBCO), supplemented with 10% FCS, Na-Pyruvate and antibiotics. The EBV-infected cell lines Raji, B95.8, IB4 and P3HR1 as well as 293-T cells were previously described (Barth et al., 2003; Grässer et al., 1993; Voss et al., 2001). Mutu I and Mutu III cell lines were a kind gift from Prof. Martin Rowe, Birmingham, UK (Sample et al., 1991). Mutu I does not express EBNA2 while Raji, Mutu III and B95.8 contain EBNA2. P3HR1 contains a non-transforming virus that has a deletion in the EBNA2 gene. The metabolic labelling of B95.8 cells with  $\text{H}_3^{32}\text{PO}_4$  (Hartmann, Braunschweig, Germany) and the analysis of precipitated EBNA2 by SDS-PAGE and autoradiography was carried out as described (Grässer et al., 1992).

#### Transfections

For transient expression of EBNA2 proteins,  $5 \times 10^6$  293-T cells were transfected with 8  $\mu$ g/10 cm dish of the pSG5-EBNA2-wt or pSG5-EBNA2- $\Delta$ RG expression vectors (Barth et al., 2003) using Nanofectin® (PAA, Cölbe, Germany). Western blotting by the ECL®-method (GE Healthcare, München, Germany) was carried out as described (Barth et al., 2003).

#### Preparation of native whole cell extract

Raji or B95.8 cells were treated for 72 h with the methylation inhibitor AdOx (Sigma, München, Germany) at 20  $\mu$ M. Lytic viral replication was induced by treatment of the cells for 48 h with 32 pM of the phorbol ester TPA (Barth et al., 2008) and cells were lysed for 30 min on ice in buffer 1 (PBS supplemented with 0.5% IGEPAL (Sigma) and 0.15 M NaCl) containing protease inhibitors (Pepstatin, 2  $\mu$ g/mL, PMSF 10  $\mu$ M, Leupeptin 1  $\mu$ g/mL, Aprotinin 1  $\mu$ g/mL). The lysate was centrifuged in a tabletop centrifuge at  $15,000 \times g$  for 15 min, and the supernatant was used for further analysis.

#### Preparation of native nuclear extracts

Raji or B95.8 cells either treated with AdOx or TPA (see above) were cultivated for 72 or 48 h, respectively. Nuclear cell extracts were prepared in a buffer containing 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM  $\text{MgCl}_2$ , and 2 mM EDTA pH 8.5 (Dignam, Lebovitz, and Roeder, 1983). Gel shift analysis was carried out exactly as described (Zimber Strobl et al., 1993).

#### Immunoprecipitation

The rat monoclonal antibody (mAb) R3 (rat IgG2a) recognises a C-terminal epitope of EBNA2 (Kremmer et al., 1995) and induces a “supershift” in electrophoretic mobility shift assays (Zimber Strobl et al., 1993) while the clone 6C8 (rat IgG2a) binds to the Trp-Trp-Pro motif of EBNA2 and interferes with binding to RBPJ $\kappa$  (Sauder et al., 1994). For immunoprecipitation and gel-shift analysis, appropriate mouse or rat IgG isotype controls were used. For precipitation, 400  $\mu$ L of mAb supernatant were coupled to 100  $\mu$ L of settled protein-G-sepharose (“PGS”, GE Healthcare, München, Germany) for 1 h at 4 °C under agitation, sedimented at 5,000 rpm in a tabletop centrifuge and washed once with 1 mL of lysis buffer 1. For precipitation experiments either 400  $\mu$ g protein of native whole cell extract or 100  $\mu$ g protein of native nuclear extract was added and incubated for 2 h at 4 °C under agitation, washed three times with lysis buffer 2 (PBS with 0.5% IGEPAL and 0.5 M NaCl) and once with lysis buffer 1. The pellet was resuspended in  $2 \times$  SDS sample-buffer and incubated for 10 min at RT or heated at 98 °C. For Western blot analysis using the aDMA-specific antibody 6F12, cell extracts or precipitated material was resuspended for up to 3 h in sample buffer without  $\beta$ -mercaptoethanol at RT without heating. Peptide inhibition of the aDMA-specific antibody in Western blot analysis was carried out by incubating 300  $\mu$ L of 6F12 tissue culture supernatant with 20  $\mu$ g of peptide dissolved in 10  $\mu$ L PBS for 1 h prior to addition of the antibody solution to the Western blot. Inhibition of the sDMA-specific antibodies in immunoprecipitation experiments, 300  $\mu$ L of 13B10 antibody solution was bound for 2 h to protein G Sepharose (PGS), then 20  $\mu$ g peptide in 20  $\mu$ L of PBS was added and incubated for 18 h at 4 °C. The PGS was then washed and used for immunoprecipitation.

#### Sucrose-gradient centrifugation

Native nuclear extract (200  $\mu$ L) was loaded on a 5–40% sucrose-gradient (10 mM HEPES pH 7.9, 5 mM  $\text{Na}_3\text{PO}_4$ , 5 mM KCl, 0.5 mM  $\text{MgCl}_2$ , 1 mM DTT) and centrifuged at  $265,000 \times g$  for 6 h at 4 °C. Typically, 17 fractions were collected (Grässer et al., 1991) and either concentrated by ethanol-precipitation prior to Western blotting or subjected directly to precipitation as outlined above.

#### Electrophoretic mobility shift assay

Preparation of native nuclear extracts and electrophoretic mobility shift assay (EMSA) was carried out as described above (Sauder et al., 1994; Zimber Strobl et al., 1993). The gels were documented using a PhosphorImager® (Amersham). *In vitro* transcription–translation of EBNA2 was performed using the TNT® Coupled Reticulocyte Lysate System (Promega, Mannheim, Germany) as described (Barth et al., 2003) following the instruction of the manufacturer. Typically, 50  $\mu$ L of the transcription–translation mix were programmed with 1  $\mu$ g of vector DNA using T7 RNA polymerase. For *in vitro* generation of RBPJ $\kappa$  in this system, we used the vector AJ247 (Maier et al., 2005) kindly supplied by B. Kempkes, Helmholtz Zentrum München. To generate non-methylated protein, EBNA2 was *in vitro* transcribed–translated using the *E. coli*-based “Rapid Translation System” (RTS 500®, Roche). For this purpose, the coding region of EBNA2 was PCR-amplified using the primers EBNA2-5'Nde (5'-ccg gaa ttc cat atg cct aca ttc tat ctt gc) and EBNA2-3'Sal (5'-gcg aat tgc tgc act tac tgg atg gag ggg cga g). The PCR product was inserted as a *NdeI*-*Sall* fragment into the pIVEX2.3-MCS vector supplied with the kit. This vector was then used to program the “RTS 500” reaction. To methylate this *E. coli*-derived EBNA2, 40  $\mu$ L of the RTS 500 reaction mixture were incubated for 2 h at 30 °C with 25  $\mu$ L of the reticulocyte lysate (see above) supplemented with 2  $\mu$ L of the  $10 \times$  reaction buffer and S-Adenosyl-Methionine (SAM) at 1.5  $\mu$ M as a methyl group donor.

## Chromatin immunoprecipitation

Mutu cells were diluted to  $5 \times 10^5$  cells/mL 24 h prior to chromatin preparation. Cells were then resuspended at  $1 \times 10^7$  cells/mL in fresh media and chromatin prepared as described previously (Bark-Jones, Webb, and West, 2006). For immunoprecipitations carried out with the anti-sDMA EBNA2 (7D9) and anti-aDMA EBNA2 (6F12) mouse antibodies, 50  $\mu$ L of protein A/G sepharose beads (Sigma) were pre-coated with 13.5  $\mu$ g rabbit anti-mouse immunoglobulins (Dako) overnight and then washed in immunoprecipitation dilution buffer (Bark-Jones, Webb, and West, 2006). Beads were then further incubated in 500  $\mu$ L immunoprecipitation dilution buffer with 100  $\mu$ L of either control, 7D9 or 6F12 hybridoma supernatant for 3 h at 4 °C with rotation. For the R3 rat monoclonal antibody, 50  $\mu$ L of protein A/G sepharose beads were directly pre-coated by incubation with 500  $\mu$ L immunoprecipitation dilution buffer and 100  $\mu$ L hybridoma supernatant. Pre-coated beads were then washed and blocked by incubation with 350  $\mu$ g single-stranded salmon testes DNA (Sigma) for 1 h at 4 °C with rotation and then re-washed. Chromatin (105  $\mu$ L) was diluted 10-fold in immunoprecipitation dilution buffer and pre-cleared using 45  $\mu$ L of pre-blocked protein A Sepharose slurry at 4 °C for 1 h. Input control samples (40  $\mu$ L) were then removed from the supernatants and stored at -20 °C and the diluted chromatin added to the antibody and salmon sperm coated beads and the immune complexes collected by rotation at 4 °C overnight. Immune complexes were then washed, and precipitated chromatin eluted and proteinase K digested as described previously (Bark-Jones, Webb, and West, 2006). DNA was purified using the QIAquick Gel extraction Kit (Qiagen) and eluted in 110  $\mu$ L sterile millipore water.

## Real-time PCR

Quantitative PCR (Q-PCR) was performed using an Applied Biosystems 7500 real time PCR machine. A series of dilutions of input control DNA (1/4, 1/16, 1/64 and 1/256) from Mutu 1 and Mutu III cells were used to create input standard curves for each primer set. Results were expressed as percentage input control following subtraction of the background signal obtained from control immunoprecipitations carried out using isotype matched control antibodies. Primers specific for the C promoter were described previously (Bark-Jones, Webb, and West, 2006). LMP1 promoter primers amplified the region from -320 to -235 relative to the LMP1 transcription start site (left primer 5' GCA GAT TAC ACT GCC GCT TC 3', right primer 5' GGC CAA GTG CAA CAG GAA 3'), the region from +31 to +117 (left primer 5' CCT GAG GAT GGA ACA CGA C 3', right primer 5' AGA GGA GGA GAA GGA GAG CAA 3') and the region from +572 to +634 (left primer 5' GGA GAT TCT CTG GCG ACT TG 3', right primer 5' TGA GCA GGA TGA GGT CTA GGA 3'). LMP2A promoter primers amplified the region from -272 to -209 relative to the LMP2A transcription start site (left primer 5' GAT AGC CTC GCG ACT CGT GGG AA 3', right primer 5' AAT CTT CAC ACA CTG CTG CTG 3'), the region from +148 to +228 (left primer 5' CCA ATA TCC ATC TGC TTC TGG 3', right primer 5' GGC TCT TCA TTA GAT TCA CGT TC 3') and the region from +379 to +470 (left primer 5' CTC ATC TCA ACA CAT ATA TGA AGA AGC 3', right primer 5' TTG ATG TGA CTT GTG ATG CAA T 3').

## Acknowledgments

We thank Ruth Nord for expert technical assistance, Richard Zimmermann, Dept. of Biochemistry, Universitätsklinikum Homburg, for the synthesis of *E. coli*-EBNA2 in the RTS 500 system, Bettina Kempkes, Helmholtz Zentrum München, for the RBPJk vector construct and Martin Rowe, CRUK Institute for Cancer Studies, Birmingham, UK for the Mutu cell lines and BZ.1 antibody. This work was supported by Deutsche Forschungsgemeinschaft (DFG) through grant KR2218/2-1 to E.K. and GR950/12-1 to F.G. and by the Wellcome

Trust through grant 078291 to M.W. We thank Lothar Strobl, Helmholtz Zentrum München, for critical reading of the manuscript. The abbreviations used are: EBV, Epstein–Barr virus; EBNA2, Epstein–Barr virus-encoded nuclear antigen 2; EMSA, electrophoretic mobility shift assay; NMA, non-methylated Arginine; sDMA, symmetrically dimethylated Arginine; aDMA, asymmetrically dimethylated Arginine; AdOx, oxidised Adenosine.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.11.023.

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# RNA Polymerase II Stalling Promotes Nucleosome Occlusion and pTEFb Recruitment to Drive Immortalization by Epstein-Barr Virus

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## Abstract

Epstein-Barr virus (EBV) immortalizes resting B-cells and is a key etiologic agent in the development of numerous cancers. The essential EBV-encoded protein EBNA 2 activates the viral C promoter (Cp) producing a message of ~120 kb that is differentially spliced to encode all EBNAs required for immortalization. We have previously shown that EBNA 2-activated transcription is dependent on the activity of the RNA polymerase II (pol II) C-terminal domain (CTD) kinase pTEFb (CDK9/cyclin T1). We now demonstrate that Cp, in contrast to two shorter EBNA 2-activated viral genes (LMP 1 and 2A), displays high levels of promoter-proximally stalled pol II despite being constitutively active. Consistent with pol II stalling, we detect considerable pausing complex (NELF/DSIF) association with Cp. Significantly, we observe substantial Cp-specific pTEFb recruitment that stimulates high-level pol II CTD serine 2 phosphorylation at distal regions (up to +75 kb), promoting elongation. We reveal that Cp-specific pol II accumulation is directed by DNA sequences unfavourable for nucleosome assembly that increase TBP access and pol II recruitment. Stalled pol II then maintains Cp nucleosome depletion. Our data indicate that pTEFb is recruited to Cp by the bromodomain protein Brd4, with polymerase stalling facilitating stable association of pTEFb. The Brd4 inhibitor JQ1 and the pTEFb inhibitors DRB and Flavopiridol significantly reduce Cp, but not LMP1 transcript production indicating that Brd4 and pTEFb are required for Cp transcription. Taken together our data indicate that pol II stalling at Cp promotes transcription of essential immortalizing genes during EBV infection by (i) preventing promoter-proximal nucleosome assembly and ii) necessitating the recruitment of pTEFb thereby maintaining serine 2 CTD phosphorylation at distal regions.

**Citation:** Palermo RD, Webb HM, West MJ (2011) RNA Polymerase II Stalling Promotes Nucleosome Occlusion and pTEFb Recruitment to Drive Immortalization by Epstein-Barr Virus. PLoS Pathog 7(10): e1002334. doi:10.1371/journal.ppat.1002334

**Editor:** Paul M. Lieberman, Wistar Institute, United States of America

**Received:** May 19, 2011; **Accepted:** September 8, 2011; **Published:** October 27, 2011

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**Funding:** This work was supported by the Wellcome Trust (grants 064014 and 078291) and by a PhD studentship to RDP from the University of Sussex. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Epstein-Barr virus (EBV) is causally associated with the development of numerous tumours including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma and post-transplant lymphoproliferative disease and immortalizes resting B cells *in vitro* generating latently infected lymphoblastoid cell-lines (LCLs) [1]. LCLs express 9 viral latent proteins: EBV Nuclear Antigens (EBNAs 1, 2, 3A, 3B, 3C and LP) and three membrane proteins (LMP 1, 2A and 2B). Following initial infection, EBNA-LP and EBNA 2 are expressed from the viral W promoter (Wp). EBNA 2 then drives promoter switching through activation of the upstream viral C promoter (Cp) to produce a long message (up to 120 kb) that is differentially spliced to produce transcripts encoding all nuclear antigens required for immortalization [2]. EBNA 2 also activates two promoters that direct transcription of the EBV oncogene latent membrane protein 1 (LMP1) and the viral LMP 2A and 2B genes [3–4]. EBNA 2 is directed to promoters via association with the cellular DNA binding proteins RBP-Jκ and PU.1 [5–8]. Transcriptional activation by EBNA 2 involves the promotion of transcription initiation through associations with histone acetyltransferases [9], chromatin remodelling complexes [10–11], and the basal transcriptional machinery

[12–14] and leads to Histone H3 and H4 acetylation at target gene promoters *in vivo* [15]. The association of EBNA 2 with target promoters is increased by asymmetric arginine dimethylation in the arginine-glycine repeat region of the protein [16] and is inhibited by phosphorylation on serine 243 during mitosis and viral lytic cycle [17–19].

The carboxy-terminal domain (CTD) of RNA Polymerase II (pol II) plays a central role in regulating efficient transcription initiation, elongation and RNA processing. It contains 52 heptapeptide repeats (Y<sub>1</sub>S<sub>2</sub>P<sub>3</sub>T<sub>4</sub>S<sub>5</sub>P<sub>6</sub>S<sub>7</sub>) and is phosphorylated largely on serines 2 and 5 during transcription [20]. Following pol II recruitment, promoter-proximal serine 5 CTD phosphorylation is mediated mainly by the TFIIF kinase, CDK7. Serine 2 CTD phosphorylation catalysed by CDK9/Cyclin T1 (positive transcription elongation factor b; p-TEFb) subsequently peaks at the 3' end of genes. Using the specific inhibitors 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and Flavopiridol, pTEFb has been shown to be required for productive elongation [21–22] by functioning as a CTD kinase and a regulator of the pol II-associated complexes DRB sensitivity-inducing factor (DSIF) and Negative Elongation Factor (NELF). DSIF and NELF induce promoter-proximal pausing that is relieved following the phosphorylation of DSIF, NELF and the pol II CTD by pTEFb



## Author Summary

Epstein-Barr virus (EBV) is associated with the development of a number of human cancers including Burkitt's lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma and post-transplant lymphoproliferative disease. The virus infects B cells rendering them immortal through the production of a small number of viral proteins in the latently infected cell. Many of the viral proteins required for B-cell immortalization are produced from a very long protein-coding RNA message that initiates at the main viral latency promoter C, and our results provide important new information on how this message is produced. Specifically we show that the production of this long RNA is driven by the recruitment of the elongation factor (pTEFb) to paused transcription complexes at the C promoter. We show that pTEFb is recruited by the chromatin-associated protein, Brd4. Treatment of cells with a recently developed Brd4 inhibitor and inhibitors of the pTEFb elongation factor inhibits production of transcripts derived from the long EBV message thereby highlighting Brd4 and pTEFb inhibitors as potential anti-EBV agents.

[23–26]. Although NELF is localized to promoters and promoter-proximal regions *in vivo*, DSIF, in its phosphorylated form, continues to associate with pol II throughout genes and functions as a positive elongation factor capable of reducing polymerase stalling at pause sites, preventing transcript release and stimulating elongation [25,27]. Interestingly, although initial studies in *Drosophila* documented the negative effects of NELF-induced promoter-proximal pausing on transcription of a subset of genes, including the Hsp70 locus [28–29], recent studies have demonstrated that the presence of NELF is required for the efficient transcription of the majority of *Drosophila* genes, forming a barrier to nucleosome assembly around the promoter [30].

Although pTEFb activity may be required for the efficient transcription of many cellular genes [22], not all gene transcription is pTEFb-dependent [31–32] and it is clear that many cellular and viral transactivators recruit and/or activate pTEFb to facilitate high-level gene-specific transcription elongation [33]. The bromodomain protein Brd4 can also recruit pTEFb to promoters via acetylated histones [34–35].

We have shown that EBNA 2 transcriptional activation requires pTEFb activity and promotes serine 5 CTD phosphorylation [36]. In this study we investigated how long-range EBV transcription required for viral immortalization is driven from the EBNA 2-responsive C promoter. Our results provide the first demonstration that significant levels of pol II accumulate specifically at Cp in association with the pausing factors DSIF and NELF and that pTEFb is recruited to the promoter at high level. Our data indicate that promoter-proximal pol II accumulation at Cp is directed by specific DNA sequences and maintains nucleosome depletion. Pausing facilitates pTEFb recruitment via Brd4 to drive high-level serine 2 CTD phosphorylation to promote production of the EBNA-encoding transcripts required for EBV immortalization.

## Results

### EBNA 2 Stimulates CTD Phosphorylation on Serine 2 and Serine 5 at Distal Genome Regions

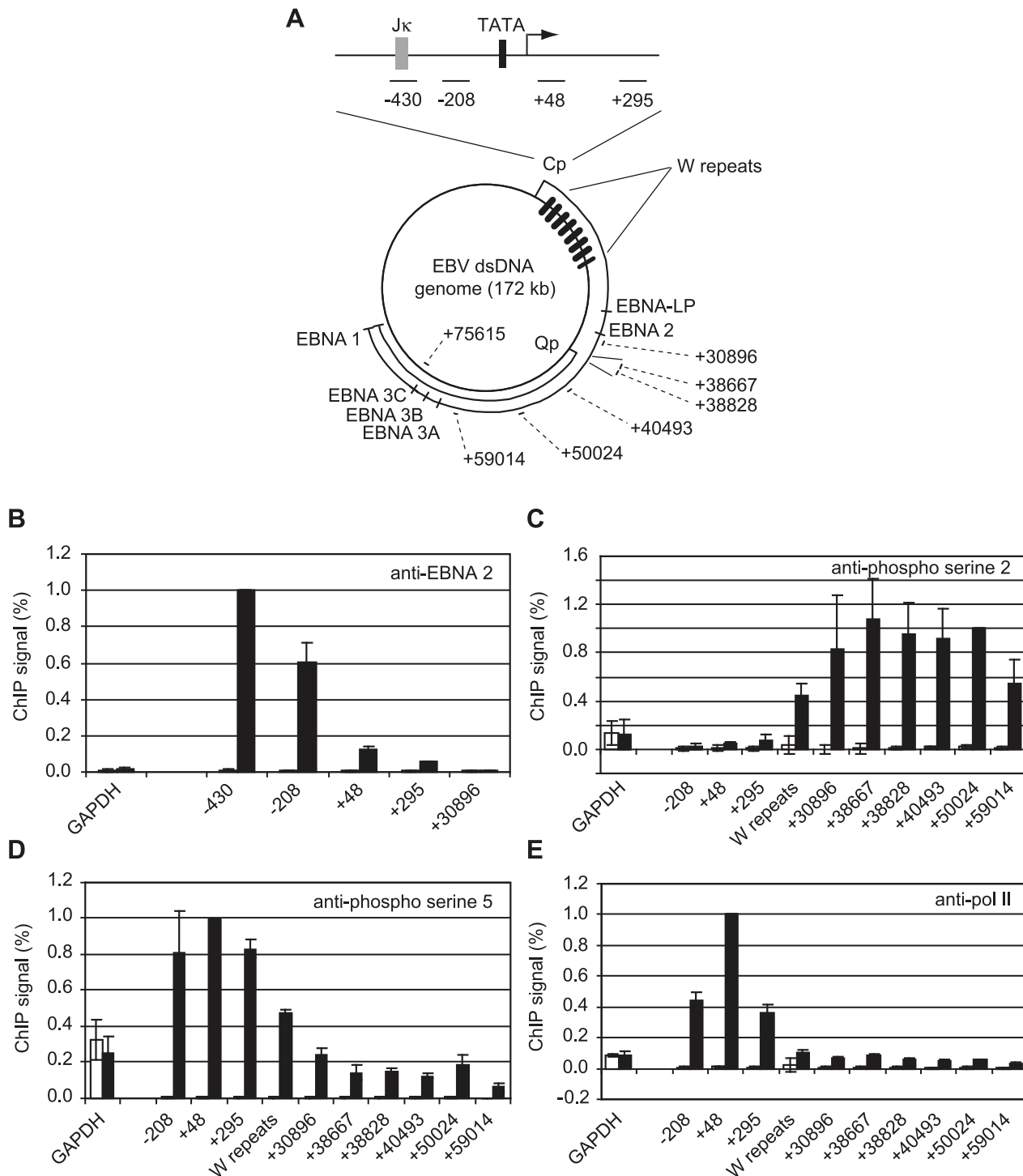
We have previously demonstrated that EBNA 2 increases serine 5 CTD phosphorylation at viral latency promoter (Cp)-proximal and downstream regions and requires pTEFb for activation of both Cp and LMP1p [36]. To examine the role of EBNA 2 in facilitating

transcription of the very long primary transcript (~120 kb) encoding all EBV nuclear antigens (EBNAs) from Cp, we probed EBNA 2-driven changes in CTD phosphorylation at distal genome regions. We examined Cp transcription in a pair of EBV-positive Burkitt's lymphoma (BL) clonal cell-lines that either maintain the original EBNA 1-only (Latency I) BL tumour phenotype (Mutu I) or have drifted in culture to express the full panel of latent antigens including EBNA 2 (Mutu III) [37]. We found that EBNA 2 binding to Cp peaked around the RBP- $\kappa$  site in Mutu III cells and was undetectable in Mutu I cells as expected (Figure 1B). EBNA 2-activated transcription in Mutu III cells resulted in increased serine 2 CTD phosphorylation which was evident from +295 and started to increase significantly in the W repeat region of the genome (typically 7.6 repeats located +666 to +24020 downstream from Cp), remaining high up to approximately 60 kb downstream (Figure 1C). In line with our previous observations in cells expressing conditionally active EBNA 2 [36], we found that EBNA 2-activated Cp transcription resulted in large increases in pol II recruitment and serine 5 CTD phosphorylation at promoter-proximal regions consistent with the promotion of transcription initiation (Figure 1D and E). In this study we found that increased serine 5 CTD phosphorylation was maintained at distal regions (Figure 1D). ChIP assays using an antibody that precipitates total pol II detected increases in the association of pol II with distal regions in Mutu III cells compared to Mutu I cells (Figure 1E), consistent with the promotion of transcriptional elongation by EBNA 2 to drive synthesis of the full panel of EBNAs expressed in Mutu III cells. Importantly, the observed changes in distal pol II CTD phosphorylation could not be accounted for by increased pol II presence alone, since increases in phospho-epitope levels exceeded the increases in total pol II (Figure 1E). We confirmed that distal serine 2 CTD phosphorylation required functional EBNA 2 using cells expressing a conditionally-active estrogen receptor-EBNA 2 fusion protein [38]; high level serine 2 CTD phosphorylation was detectable up to 75 kb downstream from Cp only in the presence of beta-estradiol (Figure S1).

Interestingly, our experiments revealed a large peak of pol II accumulation at Cp consistent with significant pol II stalling despite the fact that Cp was constitutively active (Figure 1E). In contrast, high levels of pol II were not detectable at the alternative promoter Q (Qp, +38800 downstream from Cp) that drives EBNA 1 transcription in Mutu I cells (Figure 1A & E), despite the fact that Q was fully active (Figure S2), indicating a lack of high-level pol II recruitment or stalling at Qp.

### pTEFb Is Recruited to Cp and Is Required for Distal Pol II CTD Phosphorylation

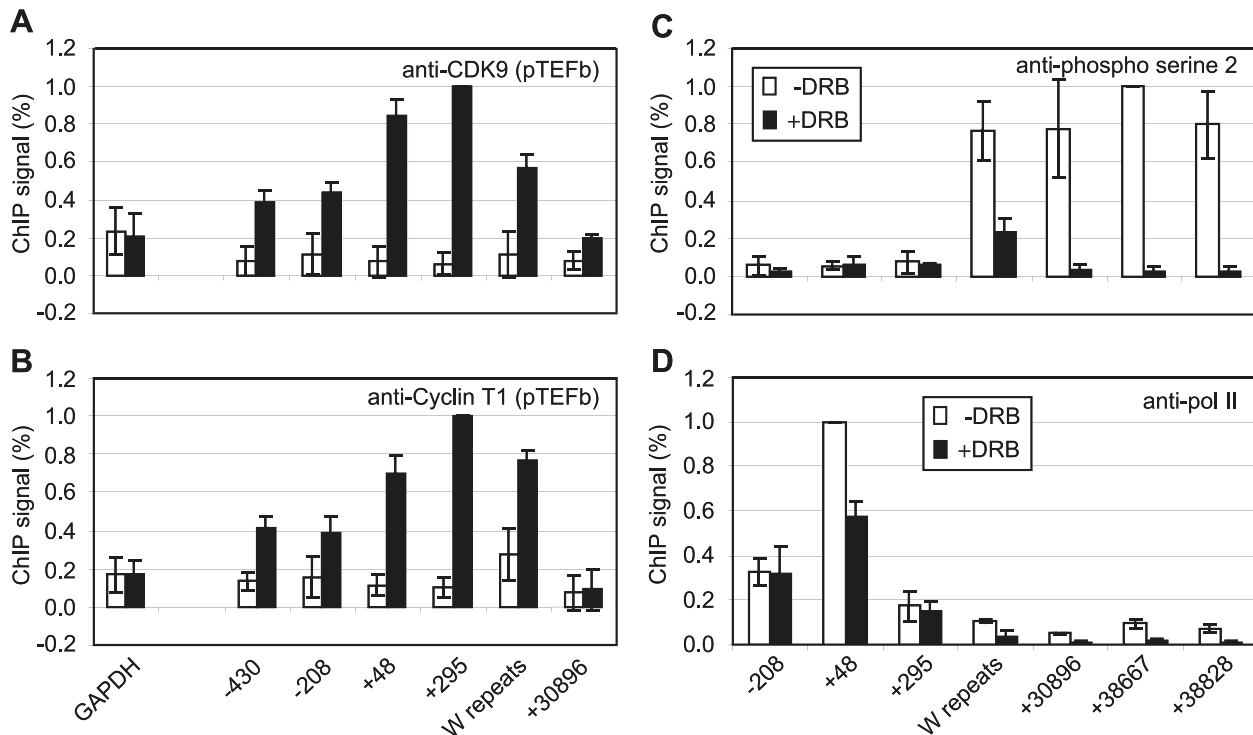
We have previously demonstrated that EBNA 2-activated transcription requires pTEFb activity [36]. Since CDK9 predominantly phosphorylates the pol II CTD on serine 2 during elongation through association with the travelling pol II complex [39], we examined pTEFb recruitment at Cp. ChIP assays using anti-CDK9 and anti-cyclin T1 antibodies demonstrated that high levels of both subunits of pTEFb were associated with Cp in Mutu III cells (Figure 2). Consistent with a role for pTEFb in distal serine 2 CTD phosphorylation, pTEFb was detectable in the W repeats and at 31 kb downstream (Figure 2) but fell to levels below the limits of detection of our ChIP assays thereafter. Previous studies have shown that pTEFb levels can drop significantly and be barely detectable using standard ChIP methods even 2 kb downstream from promoters, despite clear evidence of pTEFb function (i.e. Serine 2 phosphorylation) at these regions [40–41]. To further confirm that pTEFb was the kinase responsible for pol II CTD phosphorylation beyond 31 kb, Mutu III cells were treated with the pTEFb inhibitor,



**Figure 1. High-level pol II accumulation at Cp and CTD phosphorylation at distal EBV genome regions.** (A) Diagram showing the locations of the amplicons generated by the indicated primer sets (Table S1) at the C promoter and around the circular EBV episome. Numbers indicate the 5' end of the forward primer relative to the Cp transcription start site in the annotated B95-8 EBV sequence (NC\_007605.1). The RBP-Jk site (grey box) and TATA box (black box) are shown. (B) ChIP using anti-EBNA 2 antibodies. Percentage input signals, after subtraction of no antibody controls, are expressed relative to the highest signal obtained in all or the majority of experiments. Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments carried out using at least 2 chromatin batches from Mutu I cells (open bars) and Mutu III cells (black bars). ChIP using anti-phospho serine 2 pol II CTD antibodies (C), anti-phospho serine 5 pol II CTD antibodies (D) and anti-pol II antibodies (E). doi:10.1371/journal.ppat.1002334.g001

DRB. Our results demonstrated that DRB ablated serine 2 phosphorylation on the pol II CTD and severely reduced polymerase retention at distal regions (Figure 2). We also observed

a reduction in pol II phosphorylation on serine 5 at distal regions, supporting previous observations of a role for pTEFb in catalysing serine 5 phosphorylation during elongation (Figure S3) [39]. ChIP



**Figure 2. pTEFb is recruited to Cp and is required for serine 2 phosphorylation.** Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments carried out using at least 2 chromatin batches. Mutu I signals (open bars) are compared to Mutu III signals (black bars) in ChIP using anti-CDK9 antibodies (A) or anti-cyclin T1 antibodies (B). (C) ChIP using anti-phospho serine 2 pol II CTD antibodies in Mutu III cells minus (open bars) or plus 500  $\mu$ M DRB (black bars). (D) ChIP using anti-pol II antibodies in Mutu III cells  $-/+$  DRB. doi:10.1371/journal.ppat.1002334.g002

for Tata box binding protein (TBP) confirmed that DRB treatment did not have general non-specific effects on Cp pre-initiation complex assembly (Figure S3). DRB treatment of cells expressing conditionally active EBNA 2 also confirmed the requirement for pTEFb for distal serine 2 CTD phosphorylation and pol II retention up to 75 kb downstream during EBNA2-dependent transcription (Figure S3).

### High-levels of the Pausing Complexes NELF and DSIF Are Present at Cp

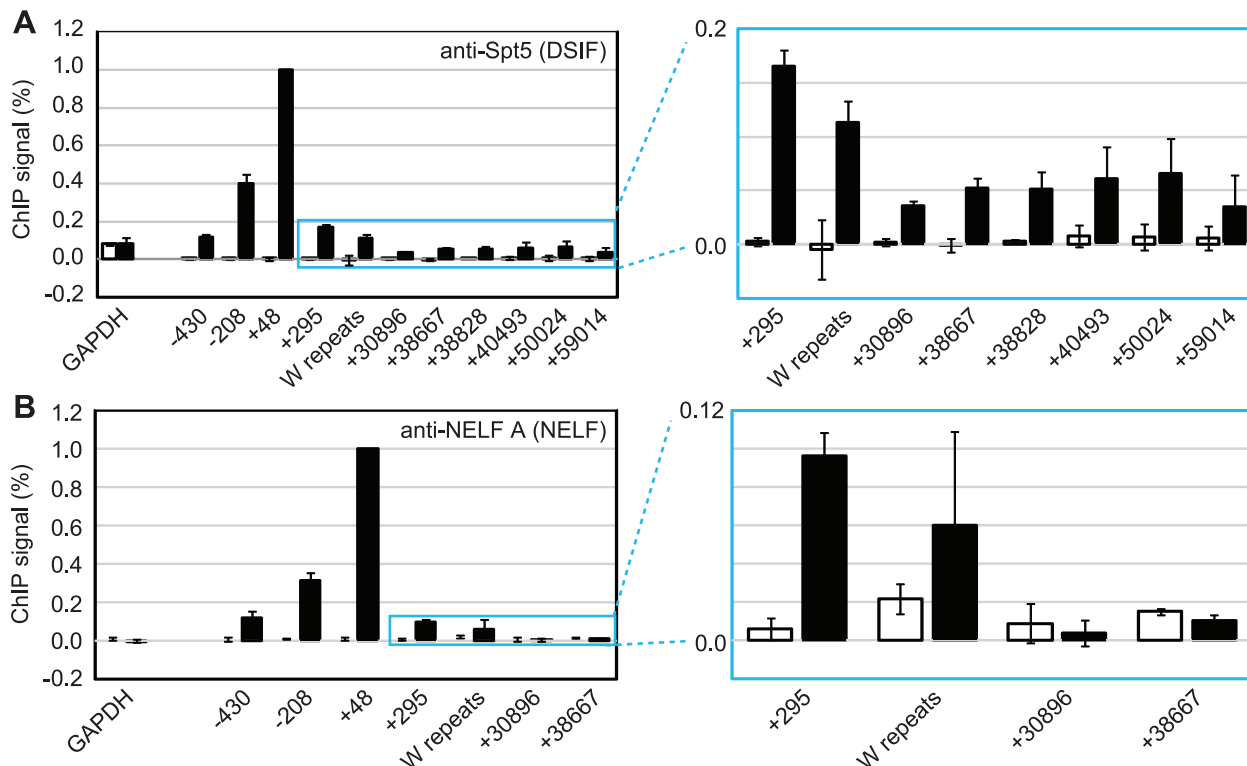
To further investigate pol II stalling at Cp, we examined the association of the pausing complexes NELF and DSIF with the promoter. We detected high-levels of the NELF-A subunit of the NELF complex and the Spt5 subunit of the DSIF Spt4-Spt5 heterodimer at Cp in Mutu III cells (Figure 3) consistent with DSIF and NELF-induced polymerase stalling. Unlike NELF, which was absent from distal regions of the template, Spt5 remained detectable at distal regions consistent with a role for DSIF in promoting transcriptional elongation [25,27] (Figure 3). EBNA 2-dependent pausing complex recruitment to Cp was confirmed in cells expressing conditionally active EBNA 2 (Figure S4). Our results suggest that recruitment of pTEFb to Cp is likely to be required to overcome stalling induced by DSIF and NELF and promote elongation to distal regions through serine 2 phosphorylation of the pol II CTD.

### Low Level Pol II CTD Serine 2 Phosphorylation and pTEFb, NELF and DSIF Recruitment at the EBNA 2-regulated LMP Gene Locus

To determine whether polymerase stalling, high level pTEFb recruitment and large increases in serine 2 CTD phosphorylation

were evident at other shorter EBNA 2-responsive transcription units, we performed ChIP assays using primers specific for the EBNA 2-activated LMP genes (Figure 4). Transcription of the LMP2A gene is regulated by EBNA 2-via two RBP-J $\kappa$  sites; EBNA 2-dependent LMP1 transcription is driven by a bidirectional promoter located in the reverse orientation in the EBV genome via EBNA 2 binding to both RBP-J $\kappa$  and PU.1 (Figure 4A). This bidirectional promoter also drives transcription of the LMP2B gene. The LMP2A and LMP1 transcription units therefore overlap and ChIP assays with primer sets 3–8 detect transcription complexes associated with either or both genes (Figure 4A). ChIP assays detected the same or higher levels of EBNA 2 binding to the LMP1 and LMP2A promoters in Mutu III cells to those detected at Cp (Figure 4B). Interestingly however, pTEFb recruitment to LMP promoters was barely detectable and no pol II stalling was evident at either LMP promoter (Figure 4). To rule out the possibility that we had failed to detect a pol II peak at LMP2Ap due to the location of our primer sets (–268 to –185 and +150 to +231), we designed an additional primer set spanning the transcription start site (–50 to +34). This primer set did not detect any higher pol II signal than the flanking primer sets (Figure S5). NELF and DSIF recruitment to the LMP locus was also minimal (Figures 4 and S6). Consistently, pol II CTD serine 2 phosphorylation did not reach the high levels observed at distal Cp regions and serine 5 phosphorylation on the pol II CTD was also much reduced (Figure S6). Similar results were obtained when Cp and the LMP locus were compared in an EBV-infected LCL (Figure S7).

To exclude the possibility that low-level pol II and transcription factor association with the LMP gene locus simply reflected low-levels of LMP transcription in the cell-lines under study, we used



**Figure 3. DSIF and NELF are recruited to Cp in Mutu III cells.** Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments carried out using at least 2 chromatin batches. Mutu I signals (open bars) are compared to Mutu III signals (black bars). ChIP using anti-Spt5 antibodies to detect DSIF (A) or anti-NELF-A antibodies to detect NELF (B). Blue-edged graphs show zoomed-in sections to allow better visualization of downstream primer signals.  
doi:10.1371/journal.ppat.1002334.g003

real-time PCR to determine the levels of Cp-initiated EBNA 2 and EBNA 1 transcripts and compared these to levels of LMP1 transcripts in Mutu III cells and two EBV-infected LCLs (Figure S8). We found that LMP1 transcript levels were equivalent to the levels of Cp-initiated EBNA 2 and EBNA 1 transcripts produced in the same cell-line, although there were variations in the level of transcripts produced between cell-lines probably as a result of differences in EBV genome copy number (Figure S8).

Taken together, our data indicate that pol II accumulation and high-level pTEFb recruitment is not a general characteristic of EBNA2-activated promoters, but is specific to Cp. Moreover, the level of promoter-associated pol II does not simply reflect the level of gene transcription from Cp and LMP1p.

### Pol II Stalling at Cp Maintains a Nucleosome-depleted Region

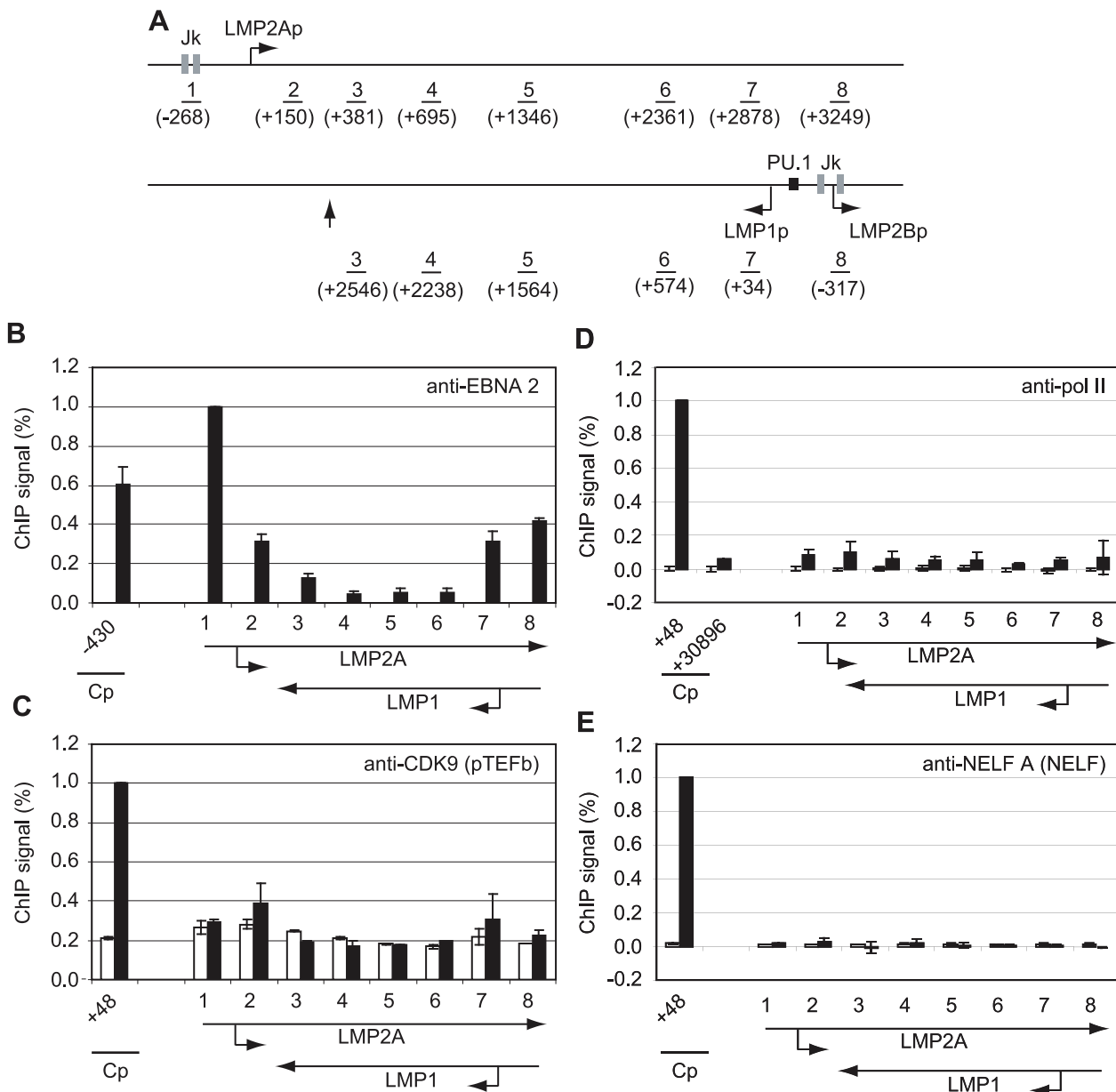
Pol II stalling has recently been implicated in the promotion of gene activity through the maintenance of a promoter-proximal nucleosome-free region [30]. We therefore investigated whether the region around Cp was depleted of nucleosomes in the presence of stalled polymerase in Mutu III cells and an LCL where Cp is active, compared to Mutu I cells where Cp is inactive. Nucleosome levels were measured in ChIP assays using antibodies against the core histone, histone H3 [41]. Strikingly, we detected an 84% decrease in nucleosome occupancy at Cp in Mutu III cells compared to Mutu I cells using primer sets that spanned the region  $-208$  to  $-96$  bp upstream of the transcription start site and a 78% and 73% decrease with primer sets spanning regions  $+48$  to  $+167$  and  $-430$  to  $-337$ , respectively (Figure 5). Nucleosomes were

similarly depleted from these regions in an EBV-infected LCL (Figure 5). In contrast, levels of nucleosome depletion at similar regions around LMP2Ap and LMP1p were much lower, consistent with the absence of stalled pol II at these promoters (Figure 5). It is therefore clear that in the absence of Cp activity in Mutu I cells, nucleosomes assemble over promoter regions, but in the presence of stalled polymerase in Mutu III cells, Cp is maintained in a nucleosome-depleted state. In contrast, the low levels of pol II initiating at the LMP promoters are unable to maintain a highly nucleosome-depleted region and transient remodelling is likely to facilitate initiation.

### Pol II Stalling at Cp May Be Directed by Specific Sequences that Allow Increased Access to the Transcription Machinery

Gene-specificity of polymerase stalling may be directed by the ability of promoters to recruit high levels of the general transcription factor TFIID and thus high levels of polymerase molecules [42–43]. Promoters that contain DNA sequences less favourable for nucleosome assembly may therefore be predicted to recruit TFIID and transcription complexes more efficiently and accumulate stalled pol II in association with DSIF and NELF. To test whether this could explain the specificity of polymerase stalling at Cp, we examined the propensity of the DNA sequences around Cp to assemble into nucleosomes using a nucleosome occupancy prediction program [http://genie.weizmann.ac.il/software/nucleo\\_prediction.html](http://genie.weizmann.ac.il/software/nucleo_prediction.html) [44]. This revealed a dramatic difference in the probability of nucleosome occupancy at Cp compared to the LMP promoters (Figure 6). The region of Cp encompassing the

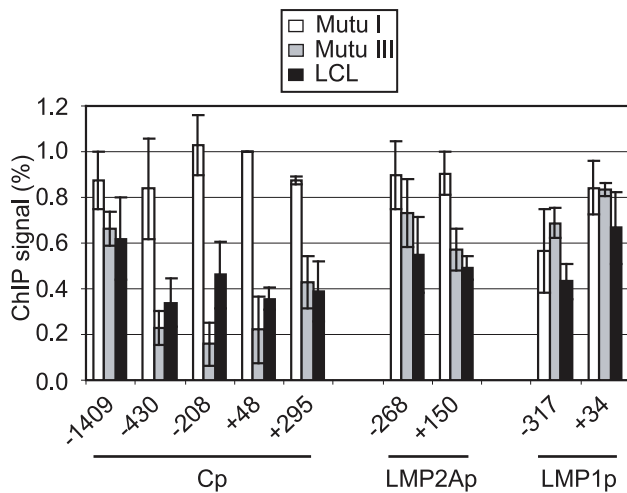




**Figure 4. Pol II is not paused at the LMP gene loci and little pTEFb is recruited.** (A) Diagram of the LMP gene locus showing the locations of the amplicons generated by the indicated primer sets (1–8). Numbers indicate the 5' end of the forward primer relative to the starts of the LMP1 or LMP2A mRNA sequences (bent arrows) in the B95-8 annotated EBV sequence (NC\_007605). The RBP-Jκ sites (grey boxes) and PU.1 site (black box) are shown. The vertical arrow indicates the position of the LMP1 polyadenylation site. Results show the mean  $\pm$  standard deviation of a minimum of two independent experiments using Mutu I (open bars) and Mutu III cell chromatin (black bars). Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using Cp-specific primers. ChIP using anti-EBNA 2 antibodies (B), anti-CDK9 antibodies (C), anti-pol II antibodies (D) and anti-NELF-A antibodies (E).  
doi:10.1371/journal.ppat.1002334.g004

TATA signal appears much less likely to be occupied by nucleosomes compared to the equivalent regions of LMP1p and LMP2Ap (TATA boxes are located at  $-31$  to  $-26$ ,  $-32$  to  $-27$  and  $-28$  to  $-23$  at the C, LMP1 and LMP2A promoters respectively). Consistent with these predictions, ChIP assays using an anti-TBP antibody detected dramatically lower levels of TBP binding at the LMP promoters compared to Cp (Figure 6). We detected high-level TBP association around the Cp TATA box ( $-107$  to  $-2$ ) and upstream ( $-208$  to  $-96$ ) presumably as a result of cross-linked interactions between TBP (TFIID) and the

transcription complex following initial TBP binding to the more accessible TATA signal. Our data are therefore in agreement with a model in which initial recruitment of high levels of pol II to Cp, presumably in association with the pol II binding factors NELF and DSIF, is driven by increased accessibility of the promoter to TBP. It is clear however, that in the absence of active Cp transcription in Mutu I cells, nucleosomes are able to assemble at Cp (Figure 5) and that the reduced probability of nucleosome occupancy may provide an initial advantage to pre-initiation complex assembly, but does not completely preclude nucleosome

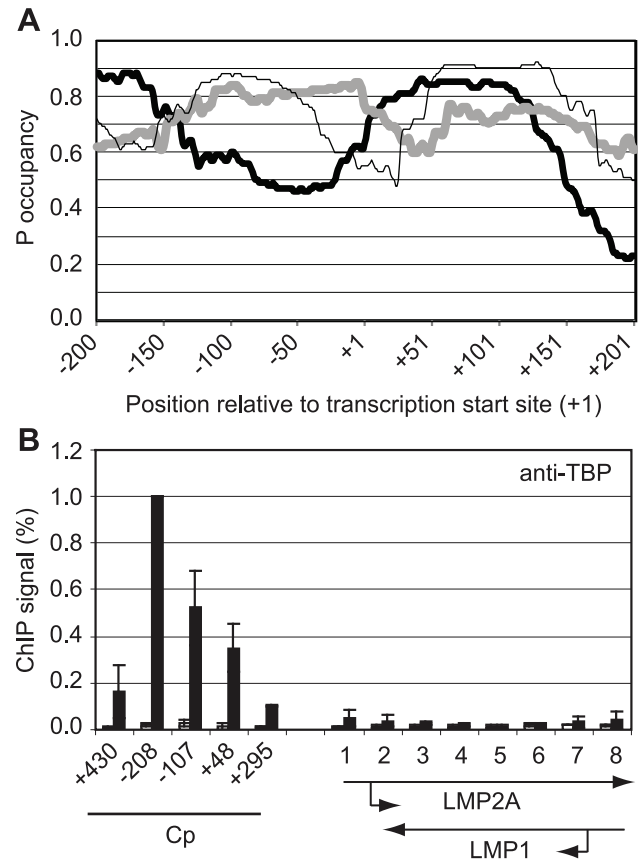


**Figure 5. Pol II pausing at Cp prevents nucleosome assembly around the promoter.** ChIP using anti-histone H3 antibodies. Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments carried out using at least 2 chromatin batches in Mutu I cells (open bars), Mutu III cells (grey bars) and the PER 253 B95-8 LCL (black bars). Primer sets probed promoter-proximal regions of Cp, LMP2Ap and LMP1p. Percentage input signals, after subtraction of no antibody controls are expressed relative to the highest signal obtained in all or the majority of experiments. doi:10.1371/journal.ppat.1002334.g005

assembly. Moreover, the presence of stalled polymerase maintains nucleosome depletion further upstream and downstream from the Cp regions predicted to be less likely to be occupied by nucleosomes since primer sets spanning  $-430$  to  $-337$  and  $+295$  to  $+406$  detect reduced histone H3 levels (Figure 5).

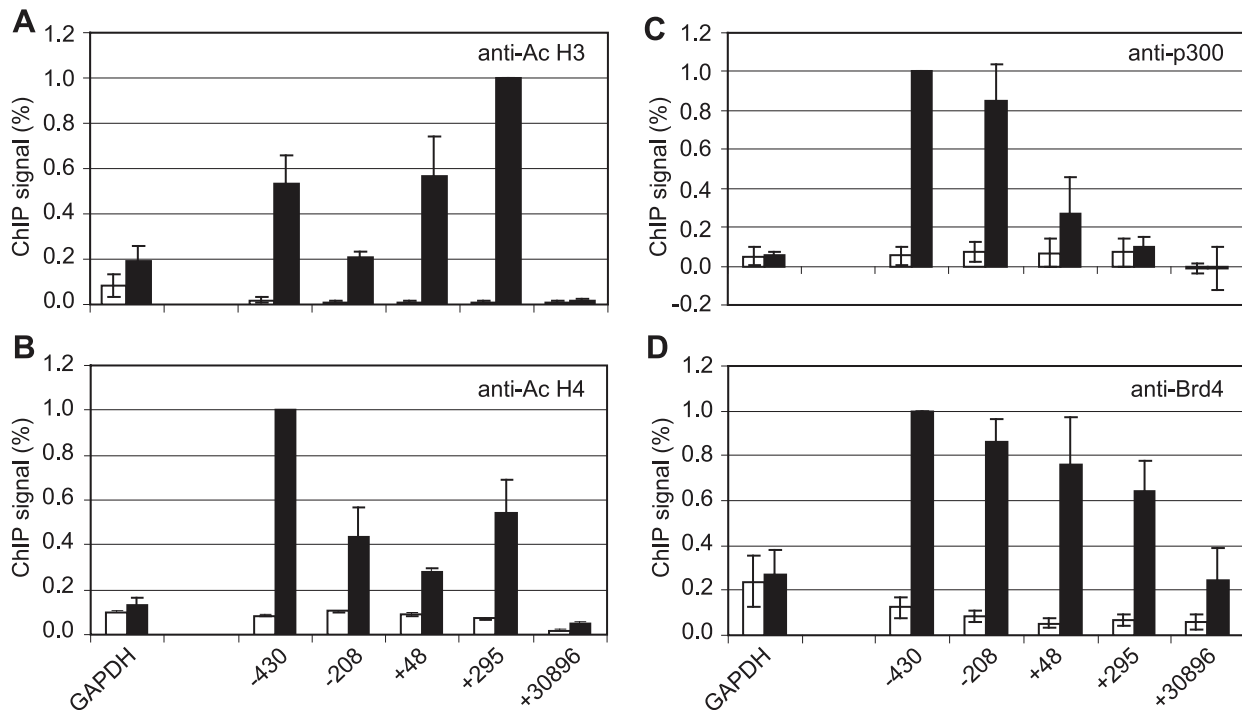
### Polymerase Stalling Stabilizes pTEFb Recruitment to Cp Via Brd4

Although pTEFb can be recruited to promoters via association with activators, we have been unable to demonstrate binding of EBNA 2 to pTEFb (data not shown). To investigate the mechanism of recruitment of pTEFb to Cp further, we examined the association of the pTEFb binding protein, Brd4, with the promoter. Brd4 is recruited via binding of its bromodomains to acetylated lysines in Histones H3 and H4 [45]. We detected large increases in Histone H3 and H4 lysine acetylation at Cp in Mutu III cells and recruitment of the Histone acetyl transferase p300, previously shown to interact with EBNA 2 [9] (Figure 7). Accordingly, Brd4 was recruited to Cp in Mutu III cells over a region spanning the peaks of histone acetylation (Figure 7). We next examined whether high-level Brd4 recruitment was Cp-specific. Our data demonstrated that Brd4 was also recruited to the LMP1 and LMP2A gene promoters consistent with the peaks of Histone H3 and H4 lysine acetylation and recruitment of p300 (Figure S9). Brd4 recruitment per se could therefore not account for Cp-specific pTEFb recruitment (Figures 2 and 4). Interestingly however, experiments carried out in the presence of cellular stress revealed that pTEFb recruitment to Cp correlates with Brd4 binding. In the presence of cellular stress, such as that induced by exposure to Actinomycin D, DRB or UV, pTEFb is released from an inactive pool, where it is complexed with 7SK snRNA and the HEXIM1 protein, as part of a stress response aimed at increasing transcription factor availability. Released pTEFb then associates with Brd4 and the levels of the pTEFb/Brd4 complex are increased [34,46–47]. DRB treatment has been previously shown to result in a 2-fold increase in the level



**Figure 6. Pol II pausing at Cp is driven by DNA sequences that promote access by TBP.** (A) The probability of nucleosome occupancy (P occupancy) at regions upstream and downstream from the transcription start site (TSS) of Cp (thick black line), LMP1p (thick grey line) and LMP 2Ap (thin black line) predicted using tools available at [http://genie.weizmann.ac.il/software/nucleo\\_prediction.html](http://genie.weizmann.ac.il/software/nucleo_prediction.html) [44]. (B) ChIP using anti-TBP antibodies analysed using Cp and LMP gene primers (see Figure 4) in Mutu I cells (open bars) and Mutu III cells (black bars). Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments carried out using at least 2 chromatin batches. Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained at Cp. doi:10.1371/journal.ppat.1002334.g006

of pTEFb/Brd4 complexes [34]. Consistent with these observations, we found that treatment of Mutu III cells with DRB resulted in a two-fold increase in both Brd4 and pTEFb recruitment to Cp indicating that Brd4 is responsible for recruiting pTEFb to Cp. Histone H4 acetylation was increased by DRB treatment at Cp, perhaps as a result of the protection from deacetylation provided by the preferential binding of Brd4 to acetylated Histone H4 residues. Previous studies have described inducible Brd4 recruitment via acetylated histone H4 but not acetylated histone H3 residues [48] and our data indicate that the pattern of Brd4 binding more closely resembles the profile of histone H4 rather than histone H3 acetylation (Figures 7 and S9). In sharp contrast, DRB treatment led to loss of Brd4 from the LMP1 promoter and decreases in Histone H3 and H4 acetylation (Figure 8G–J) (pTEFb is not detectably recruited to LMP1; Figure 4 and S5). Since the key difference between the Cp and LMP1 promoters is the presence of high levels of stalled pol II at Cp, these results suggest that pTEFb is efficiently recruited to Cp via Brd4 as a result of stable interactions between the pTEFb/Brd4 complex and the large numbers of stalled



**Figure 7. Brd4 is recruited to Cp through acetylated histones.** Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments carried out using at least two chromatin batches from Mutu I cells (open bars) and Mutu III cells (black bars). ChIP using anti-acetyl Histone H3 antibodies (A), anti-acetyl Histone H4 antibodies (B), anti-p300 antibodies (C) and anti-Brd4 antibodies (D). doi:10.1371/journal.ppat.1002334.g007

polymerases present at the promoter. Thus at LMP1p, in the absence of an accumulation of pol II molecules, pTEFb complexes brought in by Brd4 have little polymerase with which to stably associate and Brd4/pTEFb complex binding is not stabilized.

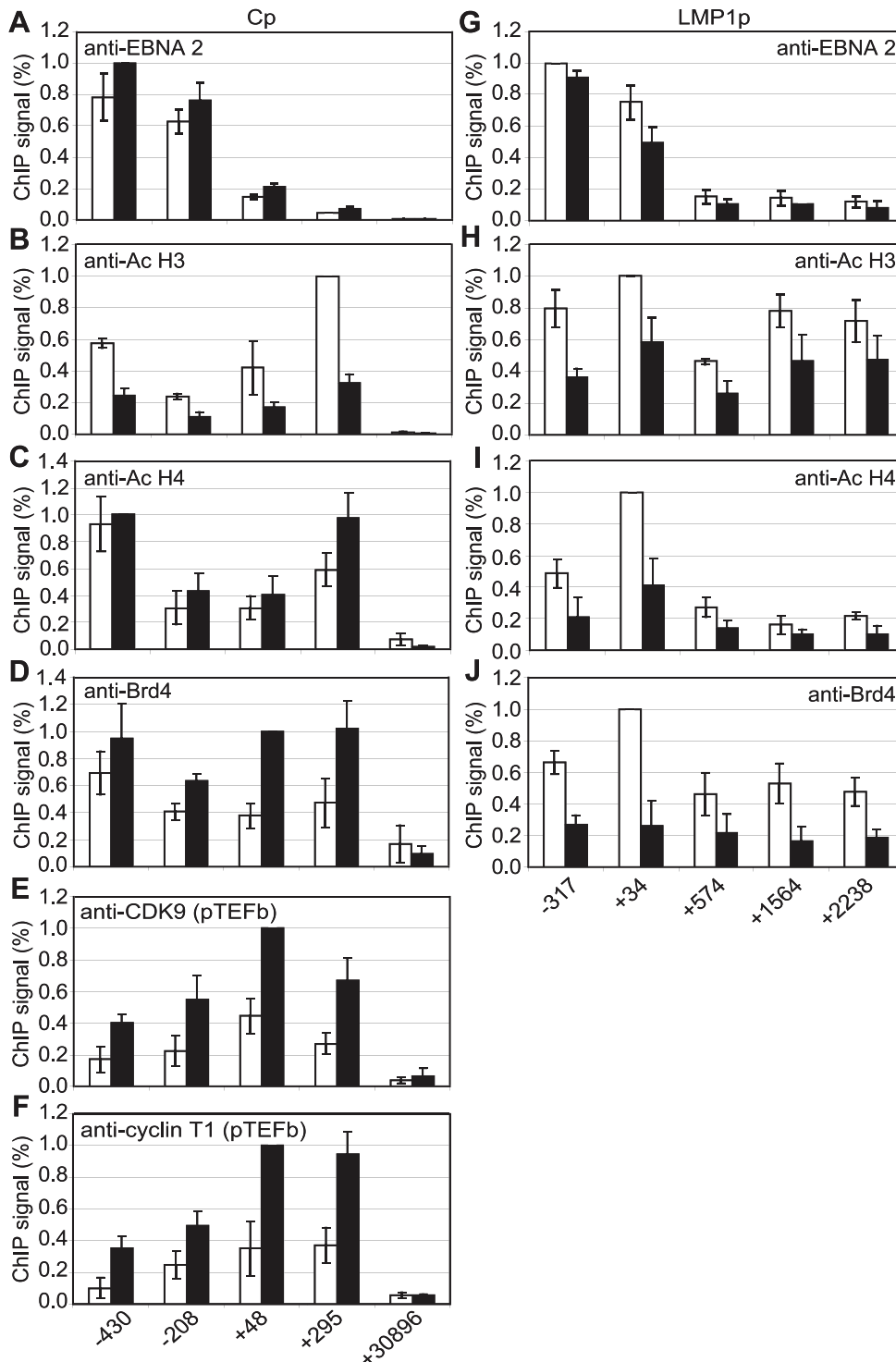
Interestingly, the essential EBV replication and transcription factor EBNA 1 has been shown to recruit Brd4 to a region of the latent origin of replication (OriP), that functions as an EBNA 1-dependent Cp enhancer [49–50]. We therefore investigated the possibility that EBNA 1 may recruit pTEFb to OriP via Brd4 and contribute to the level of pTEFb at Cp through DNA looping effects. Since EBNA 1 is expressed in Mutu I and Mutu III cells, Brd4 would be expected to be recruited to OriP by EBNA 1 in both cell types. ChIP analysis in Mutu I and Mutu III cells using primers sets close to the family of repeats (FR) element in Ori P where Brd4 was previously detected [49] revealed some Brd4 binding to Ori P in Mutu I and Mutu III cells, equivalent to that detected in the GAPDH gene (Figure S10). The level of Brd4 detected was however much lower than that present at Cp and did not appear to result in significant recruitment of pTEFb to this region of the genome (Figure S10). Our data therefore indicate that it is unlikely that pTEFb recruitment via Brd4 at OriP contributes to the level of pTEFb at Cp.

We next sought to obtain direct evidence that Brd4 binding is required for Cp but not LMP1 transcription by treating Mutu III cells with the novel small molecule Brd4 bromodomain inhibitor, JQ1, previously shown to block Brd4 association with acetylated histones [51]. Strikingly, treatment with 50 nM JQ1 for 48 hrs reduced levels of Cp-initiated EBNA 2 and EBNA 1 transcripts by 74% and 65% respectively, but had no effect on LMP1 transcript levels (Figure 9A). ChIP analysis confirmed that JQ1 dramatically inhibited Brd4 association with Cp promoter regions (Figure 9). The loss of Brd4 resulted in a significant decrease in pTEFb association with Cp,

consistent with Brd4-dependent recruitment of pTEFb to Cp (Figure 9). In summary, our data indicate that the binding of Brd4 to Cp is required for Cp transcription since it facilitates the stable association of pTEFb with the stalled polymerases present at Cp.

### Inhibition of pTEFb Selectively Inhibits Cp Transcription

Since inhibition of Brd4 binding was sufficient to selectively inhibit Cp transcription presumably through reduced pTEFb recruitment, we investigated the effects of pTEFb inhibitors on Cp and LMP transcription in Mutu III cells. We have previously demonstrated that EBNA 2 activation of both Cp and LMP1 reporter constructs was inhibited by treatment with DRB or overexpression of a dominant negative form of the pTEFb kinase, CDK9 [36]. However, our current study indicates that LMP promoters *in vivo* show little detectable pTEFb recruitment (Figure 4). Consistent with the selective high-level recruitment of pTEFb to Cp *in vivo*, we found that the pTEFb inhibitors DRB and Flavopiridol were both able to inhibit Cp transcription at concentrations at which LMP1 transcription was unaffected (Figure 10). The discrepancy between our previous results and these observations is likely explained by the fact that the promoter context in transiently transfected reporter constructs differs significantly from the appropriately assembled chromatin structures found at promoters actively engaged in transcript production in latently infected cells. Our data indicate that a reduced propensity for nucleosome assembly around Cp allows high level recruitment of TFIID and establishes polymerase pausing at the constitutively active C promoter in infected cells. These Cp-specific features may not have been established in transient assays. Thus pTEFb may be important for EBNA 2-dependent Cp and LMP promoter activity in reporter assay systems, but differences in pTEFb requirements are evident in the context of latently infected cells.

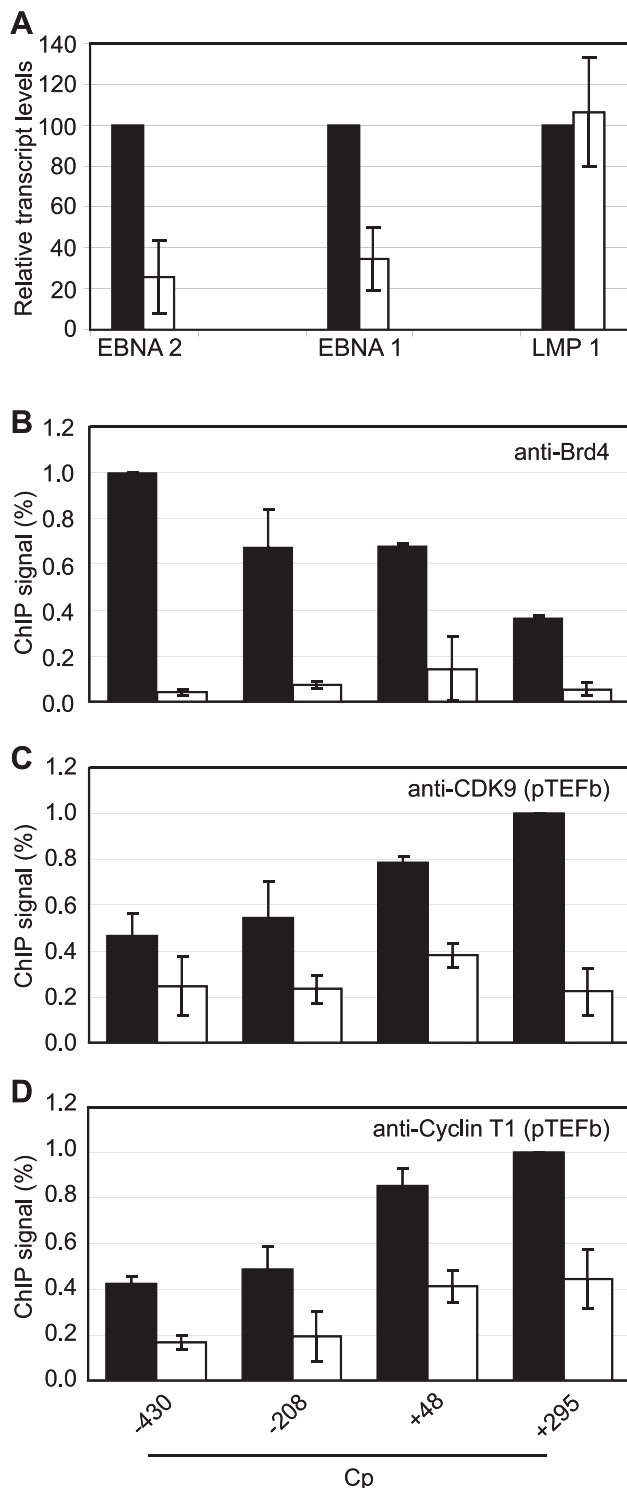


**Figure 8. Brd4 recruits pTEFb to Cp.** Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments carried out using at least 2 chromatin batches from Mutu III cells in the absence (open bars) or presence of DRB (black bars). ChIP using anti-EBNA 2 (A and G), anti-acetyl Histone H3 (B and H), anti-acetyl Histone H4 (C and I), anti-Brd 4 (D and J), anti-CDK9 (E) and anti-cyclin T1 antibodies (F). Cp analysis is shown in A–F and LMP1p/LMP 2A analysis is shown in G–J.  
doi:10.1371/journal.ppat.1002334.g008

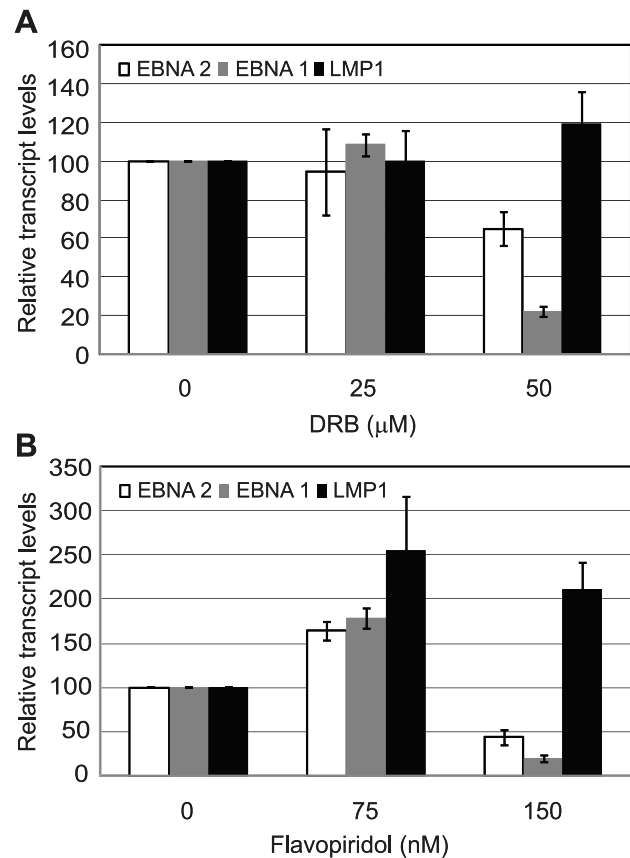
## Discussion

EBV relies on the transcription of a long polycistronic mRNA to encode the nuclear antigens (EBNAs) essential for immortaliza-

tion. Following initial production of EBNA-LP and EBNA-2 from a cellular factor-driven promoter (Wp) after primary infection [52–54], EBNA 2 activates an upstream promoter (Cp) leading to long-range transcription and the full panel of EBNA expression



**Figure 9. Brd4 is required for Cp transcription.** (A) Transcription of the Cp-initiated transcripts EBNA 2 and EBNA 1, but not the LMP1 transcript is inhibited when Brd4 binding to chromatin is blocked in the presence of the Brd4 inhibitor JQ1. Mutu III cells were treated with 50 nM JQ1 or DMSO (control) for 48 hrs and transcript levels determined using specific Q-PCR primers and actin as an endogenous control. Normalised cDNA levels are expressed relative to 48 hr control samples. ChIP using anti-Brd4 (B), anti-CDK9 (C) and anti-cyclin T1 antibodies (D) in Mutu III cells in the absence (black bars) or presence of 50 nM JQ1 for 48 hrs (open bars). Results show the mean  $\pm$  standard deviation of two independent experiments. doi:10.1371/journal.ppat.1002334.g009



**Figure 10. pTEFb inhibitors selectively reduce Cp transcription.** Mutu III cells were treated with the indicated concentrations of DRB (A) or Flavopiridol (B) for 24 hrs and transcript levels determined using the specific Q-PCR primers indicated and actin as an endogenous control. Normalised cDNA levels are expressed relative to 24 hr control samples. Results show the mean  $\pm$  standard deviation of two independent experiments. doi:10.1371/journal.ppat.1002334.g010

[2]. The molecular mechanisms behind this strategy have not been fully elucidated. Our data show that the necessity for this promoter switch goes beyond the simple advantage of utilizing a virally-controlled promoter, and may reflect a requirement to promote efficient transcriptional elongation ensuring production of the long (approximately 120 kb) primary message. Our results indicate that the specific recruitment of high levels of pTEFb to Cp in the presence of EBNA 2 is required to promote distal transcriptional elongation through serine 2 CTD phosphorylation and to overcome promoter-proximal pol II stalling induced by the high levels of the NELF/DSIF complex present at Cp. Our data suggest that Cp-specific pol II stalling may play dual positive roles in promoting transcription (i) by triggering the recruitment of pTEFb and promoting distal elongation and (ii) by maintaining a nucleosome-free region at the promoter that promotes initiation.

Our results document the presence of stalled RNA polymerase at an actively transcribing viral gene locus, unlike the situation observed at heat-shock genes, where genes temporarily in the 'OFF' state maintain promoter-proximally paused pol II to enable a rapid transcriptional 'ON' response to heat-shock that results in a re-distribution of polymerase along the gene [55]. Paused polymerase does not remain detectable when Cp is 'OFF' in the Mutu I cells used in this study because Cp is silenced in EBV positive Latency I cells through CpG DNA methylation, thus

inhibiting transcription factor binding and pol II recruitment [56–57]. Recent fine-mapping confirmed Cp methylation in Mutu I cells and demonstrated a peak of 5-methyl cytosine close to Cp that increased 7-fold in Mutu I cells compared to an LCL generated from Mutu virus, where Cp is active [58].

Significantly, we demonstrate that regulation of Cp is distinct from the regulation of the latent membrane protein promoters, where only low levels of pol II, pTEFb, NELF and DSIF are detectable and serine 2 CTD phosphorylation does not substantially increase at distal regions. The EBNA 2-dependent LMP1, LMP2A and 2B genes encode transcripts of 2.8 kb, 11.7 and 8.4 kb in length (in the B95-8 EBV genome sequence NC\_007605.1) so these shorter transcription units may therefore be less dependent on elongation factor function. It is worth noting that the LMP 2 transcription units can increase in size due the presence of varying numbers of ~500 bp terminal repeat (TR) elements that are present within these genes. On entry into host cells, the EBV genome is initially in its linear form and the TR region is the site of recombination-directed genome circularization. Although the B95-8 genome sequence we have used for transcript annotation contains 4 TRs spanning 2.1 kb, TR regions of up to ~12 kb have been reported, indicating that LMP2A transcripts can be up to 25 kb in length [59]. Nonetheless, the ~120 kb Cp transcription unit requires pol II to elongate over considerably longer distances and our results indicate that it possesses distinct regulatory features that promote long-range transcription.

The specificity of pol II stalling at Cp appears to be driven by the presence of DNA sequences upstream of Cp that are less favourable for nucleosome assembly. These sequences encompass the TATA box and therefore allow increased access to TBP resulting in high-level recruitment of pol II in association with the pausing factors NELF and DSIF. Once pol II stalling is established at Cp, a more extensive region around the promoter is then maintained in a nucleosome-depleted state. We have previously demonstrated that pTEFb activity is required for the activation of both Cp and LMP1 promoter-reporter constructs by EBNA 2 [36]. In the present study, the pTEFb components cyclin T1 and CDK9 were virtually undetectable at either the LMP1/2B or LMP2A promoters. In the context of latently infected EBV immortalized cells we now show that endogenous Cp transcription can be selectively inhibited by the pTEFb inhibitors DRB and Flavopiridol at concentrations that do not affect LMP1 transcript production. It is likely that chromatin structure in our previous transient reporter constructs differs significantly from the chromatin context present in proliferating infected cells *in vivo* and thus pausing factor and elongation factor requirements at Cp may not have been faithfully recapitulated. The results presented in this manuscript show that nucleosome occupancy is likely to be the critical determinant that sets up Cp-specific polymerase pausing and pTEFb recruitment on endogenous EBV templates. It would be interesting to test whether Cp-specific regulatory features can be conferred by Cp promoter sequences alone by generating recombinant viruses in which LMP promoter regions are replaced with Cp promoter regions.

Since the studies described here have exclusively examined the nature of constitutive Cp transcription in established cell-lines, it will be interesting to investigate the kinetics of the establishment of polymerase stalling during primary B-cell immortalization, when transcription switches from Wp to Cp approximately 6 days post-infection [60], and the effects promoter switching has on CTD phosphorylation and the elongation properties of pol II. Interestingly, when we extended our nucleosome prediction analysis to include Wp, we found that the region around the

Wp TATA box has a high probability of being occupied by nucleosomes, similar to the results obtained for the LMP promoters (Figure S11). Thus, based on our findings, Wp would be less likely to accumulate stalled polymerases as a result of increased TFIID access and pol II recruitment. The switch from Wp to Cp usage may therefore be advantageous for the virus, enabling high level pTEFb recruitment and increased efficiency of elongation. It is interesting however, that Cp-deleted viruses capable of transforming B-cells have been described and more recently rare Burkitt's lymphoma cells were identified that exclusively use Wp to drive EBNA transcription [61–62]. These observations suggest that Wp may be able to achieve sufficient long-range transcription required for EBNA production during immortalization *in vitro* or in certain cell backgrounds. Importantly, the W promoter is present in multiple copies in the EBV genome (e.g. 7.6 copies in the prototype Type 1 EBV strain, B95-8) and transcription initiation from a number of W promoters may be required to generate sufficient levels of downstream transcripts. The fact that Cp deletion is a rare event however, supports the notion that Cp plays a crucial role during EBV immortalization and infected cell growth *in vivo*.

Since we detected the presence of Spt5 at distal regions, it is also possible that the recruitment of DSIF may play a positive role in Cp transcription, as documented for HIV transcription. pTEFb-mediated phosphorylation of the Spt5 subunit of DSIF at promoter-proximal regions converts DSIF into a positive-acting elongation factor that travels with polymerase to promote processivity and inhibit further pausing [25,27]. Spt5 has been shown to promote transcriptional activation by Gal4-VP16 and is recruited to the HIV-1 LTR to co-operate in the stimulation of transcriptional elongation by HIV-1 Tat [25,63]. Further experiments involving RNA interference would be useful in dissecting the roles of DSIF in the regulation of Cp transcription.

EBV strains can be classified into two virus types (1 and 2, formerly A and B) largely based on sequence differences between the EBNA 2 genes, which share only 55% homology. Despite the prevalence of type 2 viruses in Africa and their association with BL, type 2 viruses transform resting B-cells much less efficiently than type 1 strains and differences between the EBNA 2 genes appear to be the major determinant of this property [64]. Recent work has identified cellular genes that are differentially regulated by type 1 and type 2 EBNA 2 indicating that reduced gene activation by type 2 EBNA 2 may contribute to the reduced transforming potential of type 2 viruses [65]. Since type 2 EBNA 2 also initially activates the LMP1 promoter to a reduced extent [65], it is conceivable that differences in Cp control by type 1 and type 2 EBNA 2 may also be evident. However, the results described here indicate that high-level pTEFb recruitment to Cp is driven by polymerase stalling initiated by DNA sequences that promote reduced nucleosome occupancy around Cp, increasing access to TFIID, rather than through specific properties of EBNA 2 (that may vary between strains). To investigate whether type 2 Cp sequences also possessed this property, we performed nucleosome occupancy predictions using the sequence of the type 2 viral strain AG876. We found that nucleosome occupancy at the type 2 C, LMP and W promoters was predicted to be virtually indistinguishable (data not shown) from that of the type 1 B95-8 strain previously examined (Figure 6A and S11). This is perhaps not surprising given the sequence similarity between the two strains throughout most of the genome. Thus type 2 Cp sequences show the same reduced likelihood of nucleosome occupancy as type 1 viruses, compared to the respective LMP and W promoter sequences, and would be just as likely to accumulate stalled polymerases and recruit high-levels of pTEFb. It is therefore

unlikely that pTEFb recruitment would contribute to the reduced transforming potential of type 2 viruses, but further work is necessary to address this point unequivocally.

Since we have been unable to detect an interaction between EBNA 2 and pTEFb to date, we investigated alternative mechanisms for the recruitment of pTEFb to Cp. The double bromodomain protein, Brd4, has been shown to bind to the active form of pTEFb and recruit it to promoters to stimulate elongation [34–35]. Our data indicate that pol II stalling facilitates the association of Brd4-recruited pTEFb with the C promoter by providing high levels of pol II with which pTEFb can associate. In support of this hypothesis the interaction of pTEFb with Brd4 has been shown to be weak in nature and is disrupted by low salt concentrations [34]. Thus despite similar levels of Brd4 binding to Cp and the LMP gene loci, the lack of stalled pol II at LMP does not facilitate stable pTEFb binding to the transcription complex. Brd4 has been shown to play important roles in regulating viral transcription and in tethering viral genomes to chromatin [66]. Brd4 enhances HIV-1 transcription and promotes transcriptional activation of G1/S cyclin genes by murine gammaherpesvirus 68 (MHV-68) through direct interaction with MHV-68 orf73 [35,67]. Brd4 also plays an important role in the repression of human papillomavirus transcription by the viral E2 protein and tethers bovine and human papillomavirus genomes to mitotic chromosomes [68–69]. Brd4 was also recently shown to bind to the EBV latent antigen EBNA-1 and to play a role in EBNA-1 activation of transcription; knock-down or overexpression of Brd4 inhibited EBNA-1 activated transcription in reporter assays [49]. It therefore appears that Brd4 may play multiple roles in the EBV life-cycle. Our data demonstrating specific inhibition of Cp-driven EBV transcription by the novel Brd4 inhibitor JQ1 highlights the potential for drug-like derivatives of this compound as anti-EBV agents. In addition, our further evidence for the role of pTEFb in promoting EBV transcription and the inhibition of Cp transcription by pTEFb inhibitors adds weight to the possible use of the pTEFb targeting anti-cancer drug, Flavopiridol, in the treatment of EBV-associated tumours.

In summary, we demonstrate that polymerase stalling may play a role in facilitating immortalization by the tumour virus EBV. High-level recruitment of pol II and associated pausing factors to the viral C promoter maintains nucleosome depletion and necessitates pTEFb recruitment to overcome pausing. This provides high levels of pTEFb to promote the distal serine 2 CTD phosphorylation required for production of the long viral transcript encoding key EBV immortalizing genes.

## Materials and Methods

### Cell Culture

ER/EB 2.5 cells [38] were maintained as described previously [36]. Mutu I (clone 179), Mutu III (clone 48), IB4 (provided by Martin Rowe), PER 142 B95-8 LCL and PER 253 B95-8 LCL (provided by Heather Long) were cultured as described [70]. For Brd4/pTEFb inhibition experiments, Mutu cells were resuspended at  $5 \times 10^5$  cells/ml and incubated in the presence of DMSO, JQ1/SGC8001 [51] (kindly provided by Stefan Knapp, Structural Genomics Consortium, University of Oxford), DRB (Sigma) or Flavopiridol (Sigma) for 24 or 48 hours.

### ChIP Assays

ER/EB 2.5 cells were washed and resuspended at  $5 \times 10^5$  cells/ml in medium without  $\beta$ -estradiol. After 3 days  $1 \mu\text{M}$   $\beta$ -estradiol (Sigma) was added for 5 hours and chromatin prepared as described previously [36]. ER/EB 2.5 cells were treated with

$100 \mu\text{M}$  DRB (or DMSO as a control) for 2 hrs as required prior to addition of  $\beta$ -estradiol.

Mutu cells were diluted to  $5 \times 10^5$  cells/ml 24 hrs prior to chromatin preparation and resuspended at  $1 \times 10^7$  cells/ml in fresh media before crosslinking. Cells were treated with  $500 \mu\text{M}$  DRB for 2 hrs prior to chromatin preparation.

ChIP methods were optimised for each target using a number of alternative strategies.

For ER/EB 2.5 cells ChIP assays were carried out as described previously [36] by overnight incubation at  $4^\circ\text{C}$  with  $6 \mu\text{g}$  of polyclonal antibodies (anti-Pol II; N-20, anti-Spt5; H-300, Santa Cruz Biotechnology, Inc) followed by precipitation with protein A sepharose beads pre-blocked with salmon sperm DNA. EBNA 2 immunoprecipitations were carried out using  $8 \mu\text{g}$  of monoclonal antibody (PE2) and an additional incubation with secondary antibodies [36]. DNA was purified using the QIAquick Gel extraction Kit (Qiagen) and eluted in  $110 \mu\text{l}$  sterile millipore water. Phospho serine 2 immunoprecipitations in ER/EB 2.5 cells were carried out using a double-round ChIP protocol immunoprecipitating first pol II and then the phospho-specific form. Immune complexes from pol II precipitations were eluted and diluted by addition of  $850 \mu\text{l}$  IP dilution buffer. Second round immunoprecipitations were carried out using protein sepharose A/G beads (1:1 mix of protein A and G sepharose) preabsorbed first with rabbit anti-mouse IgM immunoglobulins ( $20 \mu\text{g}$ ) in  $500 \mu\text{l}$  IP dilution buffer overnight, and then with  $25 \mu\text{g}$  anti-phospho ser 2 (H5) for 3–5 hours at  $4^\circ\text{C}$ . Prior to collection of immune complexes,  $100 \mu\text{l}$  of a 50% slurry of antibody pre-coated beads were blocked using  $350 \mu\text{g}$  salmon sperm DNA for 1 hr at  $4^\circ\text{C}$ . Immune complexes were collected by rotation at  $4^\circ\text{C}$  overnight.

ChIP assays for EBNA 2 using Mutu cell chromatin were carried out as for ER/EB 2.5 cells using  $8 \mu\text{g}$  (PE2) antibodies. Pol II, Spt5, acetylated Histone H3 and acetylated Histone H4 immunoprecipitations were carried out as described previously [36] by overnight incubation of chromatin lysates with  $5 \mu\text{g}$  of anti-pol II, anti-Spt5 (H-300), anti-CDK9 (H-169), anti-Cyclin T1 (H-245), anti-Brd4 (H-250) (Santa Cruz Biotechnology, Inc), anti-acetyl-Histone H3 or H4 (Millipore) antibodies. ChIP assays for core Histone H3 were carried out using  $2 \mu\text{g}$  anti-Histone H3 antibody (Abcam, ab1791). For NELF-A, immunoprecipitations were carried out using a polyclonal antibody (Santa Cruz Biotechnology, Inc) and pre-coating protein A/G sepharose beads with  $5 \mu\text{g}$  (anti-NELF-A; A20) antibody overnight. Immune complexes were collected overnight following blocking of pre-coated beads with salmon sperm DNA as above. Phospho serine 2 and 5 immunoprecipitations using Mutu cell chromatin were carried out in a single round ChIP by pre-coating protein A/G sepharose beads with  $10 \mu\text{g}$  rabbit anti-mouse IgM overnight, prior to the addition of  $25 \mu\text{g}$  H5 or  $5 \mu\text{g}$  H14 antibodies and then salmon sperm DNA as above. All controls were treated identically but without addition of antibodies.

### cDNA Preparation

Cells were diluted to  $5 \times 10^5$ /ml, harvested after 24 hrs and total RNA extracted using TriReagent (Sigma). RNA samples were purified using the RNeasy kit (Qiagen) and cDNA was then synthesised using the ImProm II reverse transcription system using random oligonucleotides (Promega). For Brd 4 and pTEFb inhibitor experiments, cDNA was prepared from  $10^5$  cells using Power SYBR Green Cells-to-CT Kit (Applied Biosystems) according to the manufacturer's instructions.

### PCR

Quantitative PCR (Q-PCR) was performed as described previously [36] using an Applied Biosystems 7500 real-time



PCR machine (95°C for 10 mins, 40 cycles at 95°C for 15 sec and 60°C for 1 min and dissociation curve analysis). For ChIP analysis, an input control standard curve was generated for each primer set (Table S1). Generally, cDNA samples were analysed using the absolute quantitation method with standard curves generated from Mutu I or Mutu III cDNA. Transcript levels were determined using Qp or Cp-specific primers [71], cDNA-specific EBNA 2, EBNA 1 (YUK) or LMP1-specific primers [72] and either the 18S rRNA Quantitect primer assay (Qiagen) or actin primers as normalization controls (Table S1). For Brd 4 inhibition experiments, Q-PCR was carried out using Power SYBR Green Cells-to-CT Kit (Applied Biosystems) and cDNA-specific EBNA 2, EBNA 1 (YUK) or LMP1-specific primers [72] with actin as the endogenous control and analysed by Relative Quantification (ddCt).

### Immunoblotting

SDS-PAGE analysis and immunoblotting was carried out as described previously [36,70]. Blots were probed with human M.S. serum at 1/200 to detect EBNA 1 (gift from Martin Rowe), PE2 at 1/300 to detect EBNA 2 and anti-actin at 1/5000 (A-2066, Sigma). HRP-conjugated anti-mouse (Dako) or anti-rabbit antibodies (Cell Signalling Technology) were used to detect EBNA 2 and actin respectively, and HRP-conjugated protein A (1/1000, Amersham Biosciences) was used to detect EBNA 1 primary antibodies.

### Accession Numbers

The type 1 EBV genome used for primer design, transcription start sites and nucleosome predictions is the annotated sequence from the B95-8 strain (NC\_007605.1). The type 2 EBV genome used was from the AG876 strain (NC\_009334.1).

### Supporting Information

**Figure S1 Increased serine 2 phosphorylation on the pol II CTD is dependent on EBNA 2 activity.** (A) ChIP using anti-EBNA 2 antibodies in ER/EB 2.5 cells cultured in the absence (open bars) or presence of  $\beta$ -estradiol (black bars) shows functional EBNA 2 binding only in the presence of  $\beta$ -estradiol (B) ChIP using anti-phospho serine 2 pol II CTD antibodies detects high-level serine 2 phosphorylation only in the presence of  $\beta$ -estradiol. Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments carried out using at least 2 chromatin batches. (PDF)

**Figure S2 The Q promoter drives EBNA-1-only expression in Mutu I cells.** (A) ChIP using anti-acetylated histone H3 antibodies and Mutu I (open bars) and Mutu III cell chromatin (black bars). Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments carried out using at least 2 chromatin batches. Numbers indicate the 5' end of the forward primer relative to the Cp transcription start site in the annotated EBV sequence (NC\_007605.1). Qp is located at +38800 relative to the Cp transcription start site. (B) PCR amplification of Qp-specific and Cp-specific transcripts. Akata cells (Qp only) and the PER 253 LCL (Cp only) served as positive controls for Qp and Cp usage respectively. The IB4 LCL has a deletion upstream of Cp so is negative for Qp and Cp transcripts. Qp or Cp signals were normalised to 18S rRNA primer signals. (C) Western blot analysis of whole cell lysates of Mutu I and Mutu III cells. Blots were probed with M.S. human serum to detect EBNA 1, PE2 to detect EBNA 2 and re-probed with anti-actin antibodies as a loading control. (PDF)

**Figure S3 DRB treatment of Mutu III and ER/EB 2.5 cells inhibits CTD phosphorylation.** (A) ChIP using anti-phospho serine 5 pol II CTD antibodies in Mutu III cells minus (open bars) or plus 500  $\mu$ M DRB (black bars). (B) ChIP using anti-TBP antibodies in Mutu III cells  $-/+$  DRB. (C) ChIP using anti-phospho serine 2 pol II CTD antibodies in ER/EB 2.5 cells cultured in the presence of  $\beta$ -estradiol and in the absence (open bars) or presence (black bars) of 100  $\mu$ M DRB. (D) ChIP using anti-pol II antibodies in ER/EB 2.5 cells cultured in the presence of  $\beta$ -estradiol and in the absence or presence of DRB. (PDF)

**Figure S4 Pausing factor recruitment is dependent on the function of EBNA 2.** ChIP using anti-Spt5 (DSIF) antibodies in ER/EB 2.5 cells cultured in the absence (open bars) or presence of  $\beta$ -estradiol (black bars) detects significant DSIF recruitment only in the presence of functional EBNA 2. (PDF)

**Figure S5 Pol II is not paused at the LMP 2A promoter.** Results show the mean  $\pm$  standard deviation of four independent pol II ChIP experiments using Mutu I (open bars) and Mutu III cell chromatin (black bars). Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using Cp-specific primers. (PDF)

**Figure S6 Low level pol II and elongation factor recruitment at the LMP gene locus.** (A) Primers across the LMP locus are as in Figure 4. ChIP results show the mean  $\pm$  standard deviation of a minimum of two independent experiments using Mutu I (open bars) and Mutu III cell chromatin (black bars). Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using Cp-specific primers. (B) ChIP using anti-phospho serine 2 pol II CTD antibodies. (C) ChIP using anti-phospho serine 5 pol II CTD antibodies (D) ChIP using anti-cyclin T1 antibodies. (E) ChIP using anti-Spt5 antibodies. (PDF)

**Figure S7 Low level pol II and elongation factor recruitment at LMP genes in an LCL.** ChIP carried out in an EBV immortalised LCL (PER 253 B95-8 LCL). Primers across the LMP locus are as in Figure 4. Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments. Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using Cp-specific primers. (A) ChIP using anti-pol II antibodies. (B) ChIP using anti-Spt5 antibodies. (C) ChIP using anti-NELF A antibodies. (PDF)

**Figure S8 Cp-initiated EBNA 2 and EBNA 1 transcript levels are similar to those of LMP1.** Transcript levels from cDNA prepared at the same time from Mutu I, Mutu III, PER 253 B95.8 LCL and PER 142 B95.8 LCL were determined using specific Q-PCR primers to EBNA 2, Cp-initiated EBNA 1 (YUK spliced) and LMP1. Transcript quantities were determined using the absolute quantitation method and a cDNA standard curve and divided by actin quantities as a normalization control. Results show mean  $\pm$  standard deviation of Q-PCR duplicates from a representative experiment. Note that EBNA 1 transcripts initiate from Qp in Mutu I cells (Figure S2) and are not detected by the YUK EBNA 1 primer set used here. (PDF)



**Figure S9 Brd4 is recruited to LMP gene promoters.** Results show the mean  $\pm$  standard deviation of a minimum of three independent experiments using Mutu I (open bars) and Mutu III cell chromatin (black bars). LMP gene locus primers are as in Figure 4. Percentage input signals, after subtraction of no antibody controls, are expressed for comparison purposes relative to the highest signal obtained using Cp-specific primers. (A) ChIP using anti-acetyl Histone H3 antibodies. (B) ChIP using anti-acetyl Histone H4 antibodies. (C) ChIP using anti-p300 antibodies. (D) ChIP using anti-Brd 4 antibodies. (PDF)

**Figure S10 pTEFb is not recruited to OriP at high levels.** Results show the mean percentage input signal (after subtraction of the no antibody control signal)  $\pm$  standard deviation of two independent experiments using Mutu I (open bars) and Mutu III cell chromatin (black bars). (A) ChIP using anti-Brd4 antibodies. (B) ChIP using anti-CDK9 antibodies (C) ChIP using anti-cyclin T1 antibodies. Cp analysis was carried out with the primer set that gave the highest signal for each transcription factor. Ori P primers are adjacent to the EBNA 1 binding element (family of repeats, FR) (Table S1). (PDF)

**Figure S11 Nucleosome occupancy prediction analysis at Wp resembles LMP genes.** The probability of nucleosome occupancy (P occupancy) at regions proximal to Cp, LMP1p, LMP 2Ap and Wp was predicted using tools available at [http://genie.weizmann.ac.il/software/nucleo\\_prediction.html](http://genie.weizmann.ac.il/software/nucleo_prediction.html) [44]. Cp, LMP1p, LMP 2Ap and Wp TATA boxes are located at  $-31$  to

$-26$ ,  $-32$  to  $-27$ ,  $-28$  to  $-23$  and  $-31$  to  $-26$  relative to the transcription start sites (+1). (PDF)

**Table S1 Real-time PCR primers.** <sup>a</sup> Primer locations are given relative to the start of the relevant mRNA sequence in the annotated EBV sequence (NC\_007605.1) or the GAPDH gene. <sup>b</sup> Numbers relate to the annotated EBV sequence (NC\_007605.1). <sup>c</sup> Primers are located in the W repeat region which contains on average 7.6 repeats of an estimated 3072bp sequence. <sup>d</sup> Primer sequences obtained from Prof. Paul Lieberman, The Wistar Institute, Philadelphia, USA. <sup>e</sup> Primer locations for the LMP1 gene located in the reverse orientation are given in parentheses. <sup>f</sup> The LMP1 polyA is located at 166483 to 166488 so MW 361 is outside of the transcription unit. <sup>g</sup> Actin primers span exon 3 to 4. Nucleotide positions for chromosome 7 are shown (BC002409). (PDF)

## Acknowledgments

We thank Heather Long and Martin Rowe for reagents and Panagis Filippakopoulos, Jun Qi, Sarah Picaud, Stefan Knapp, Tom Heightman, Chas Bountra, Brian Marsden, Udo Oppermann, Chris Schofield, Aled M. Edwards, Cheryl H. Arrowsmith and James E. Bradner for the gift of JQ1.

## Author Contributions

Conceived and designed the experiments: MJW RDP. Performed the experiments: RDP HMW. Analyzed the data: RDP HMW. Wrote the paper: MJW.

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